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BIOCHEMISTRY

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American researchers and physicians can now judge for themselves the significance of the dramatic results of novocain therapy in maladies of old age, as reported by Dr. Anna Aslan, *Director of the Institute of Geriatrics, Bucharest, Rumania,* and her co-workers.

These seven papers, now in complete English translation, originally appeared in the West German journal *"Die Therapiewoche."* The authors report the results of large-scale clinical application of novocain injections for treatment of the sequelae of old age. The research has continued over a seven-year period and has covered more than 5,000 people. Dr. Aslan and her colleagues claim to have achieved a reversal of phenomena previously considered irreversible, e.g., in *cerebral arteriosclerosis;* they report sustained improvement in senile Parkinsonism, ichthyosis and senile Keratosis, and a reduction in arteriosclerotic symptons.

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RAPID DETERMINATION OF THE RADIOACTIVITY OF PROTEINS AND OF THE RELATIVE PROPORTIONS OF VARIOUS FRACTIONS OF RADIOACTIVE SUBSTANCES PRESENT IN LEAVES

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It is frequently necessary, in protein metabolism studies involving the use of radioisotopes, to make a series of measurements of the specific activity (Sp. Act.) of the proteins, i.e. of their radioactivity per unit weight. The measurement of the radioactivity of amounts of protein of the order of 0.1 - 1.0 mg, isolated from a small leaf or from a slice of tissue, is a simple matter, particularly if its Sp. Act. is high enough. The real difficulties are in the isolation and purification of such microamounts of protein; these difficulties are a hindrance to serial determinations involving small amounts of material, and they make it necessary to analyze pooled samples of leaves from the same level of a number of plants. However, the pooled sample method, although irreplaceable for many purposes, excludes the possibility of taking into account the individual peculiarities of leaves.

In this connection, the procedure applied by us to the preparative radioautography of leaves [1-3], whereby imprints of the leaves are made on filter paper, may be of value as applied to the analytical radiochemistry of plant tissues. This procedure has recently been applied to the determination of the specific radioactivity of leaf proteins (labelled with S^{35} or C^{14}), and of the relative proportions of various P^{32}-labelled leaf constituents.

The principle of our proposed method is as follows. In making the imprint, the protoplasm of the leaf cells is squeezed into the filter paper, free of cell-wall constituents. The leaf proteins are deposited directly on the imprint, which is washed free of nonprotein substances and lipids, and the paper is dried. Reweighing the filter paper gives the weight of proteins deposited on it, and the area of the imprint is measured. Absorption of radiation by the paper is the same for all cases, and an empirical correction is made for this factor. When these values have been determined the activity of the imprint is ready to be measured in a counter.

The surface activity of a given area of the imprint may be measured with the aid of a number of types of end-window counters, and the specific activity may hence be calculated.

One working day suffices for the performance of all the measurements involved for a series of 50-70 leaf samples (not counting time spent on calculations). At the same time there is no difficulty in processing any number of samples (at least 200-300), without necessitating the use of large amounts of chemical glassware. Determinations of the numerical ratios between various radioactive fractions, such as in work with P^{32}, is even more facilitated, since the need for weighing does not arise. A further advantage of our procedure is that the leaf imprints used can also serve for radioautographic purposes.

The method has, of course, considerable limitations, and it cannot be considered to be a substitute for the ordinary analytical methods. It can, however, be applied in researches involving serial determinations, and for the performance of rapid control estimations required during the course of a research.

EXPERIMENTAL METHODS

Preparation and processing of imprints. It is most convenient, for determinations of specific activity of leaf proteins, to use the ballistic method of preparing imprints, described in detail in our earlier publications [1-3]. By this method, the pressure applied to the leaf resting on filter paper is applied as a single blow (25 to 100 kg/cm^2). Such a blow rapidly disintegrates the leaves of many species of plants, and expels the protoplasmic contents of the cells into the filter paper. With this procedure, only one or two layers of filter paper become impregnated with the juice, since because of the very short duration of application of pressure the expelled protoplasm does not have time to soak into the lower layers of paper. Thus, the bulk of the leaf proteins are to be found in the top filter paper, and its amount can readily be determined by weighing.

The leaf imprints, prepared by this or any other method, are rapidly dried (at room temperature or in a thermostat), spread on glass sheets or enamelled trays, and sprayed with 10% trichloroacetic acid (TCA) solution. The proteins are thereby firmly fixed in the paper, so that their content is scarcely at all affected by subsequent washing. The papers are then washed free of soluble substances by rocking in a shallow dish with 2-3% TCA.

The wash solution is changed a few times. Cooled solutions are used in working with leaves containing P^{32}-labelled material, and in determinations of nucleic acids. Unlabelled compounds of sulfur and phosphorus are added to the wash solutions, in order to minimize adsorption and readsorption effects. After washing with TCA solution, the papers are washed with water, dried, placed in a beaker, and washed further in a Buchner funnel, with suction.

This very thorough washing is necessary to ensure that soluble substances are completely removed from the imprints. It should, however, be noted that some soluble compounds (methionine, phosphates) present in small amounts (0.05 - 0.8%) are so firmly adsorbed on plant proteins that it is very difficult to eliminate them from the imprints, even with the most efficient washing procedures [4]. This effect does not, however, in any way invalidate the imprinting method, since the same adsorption effect enters into any other method of analytical treatment of plant material.

After having been washed in the above way, the imprints consist of proteins, nucleic acids, and lipids. The nucleic acids can be eliminated by washing with hot, instead of cold, TCA solution; proteins are thus precipitated, while nucleic acids are dissolved out. Lipids and plastid pigments are readily eliminated by washing with alcohol, ether, chloroform, and other such solvents. This washing may be done in shallow dishes, or by extraction in Soxhlet extractors, placing 30-40 imprints rolled up together in the extraction chamber. The imprints are then dried; they now consist of unpigmented leaf proteins alone. Such imprints are then used for all the subsequent determinations.

A few tarred sheets of filter paper are subjected to all the same manipulations as are those bearing imprints, and serve as controls to show what changes take place in the weight of the paper during the washing procedures. It is most convenient to use various sorts of chromatography paper, since this has a minimum content of soluble constituents, and is of uniform thickness.

Determination of specific radioactivity of the proteins present in the imprints. The washed and dried imprints are weighed on an automatic or torsion balance. The increase in weight due to protein was from 3 to 30 mg for bean leaf imprints, depending on the size of the leaf. To this weight we add the loss in weight sustained by the control pieces of filter paper, of the same area. The sum represents the weight of proteins present in the imprint. We then measure the area of the imprint, and the weight of proteins present in an area equal to that of the window of the counter. The area of the imprints is readily measured gravimetrically. Knowing the weight and the area of the sheets of filter paper used (this was standard for a given series of experiments), we could weigh the paper remaining after cutting out the imprints, and hence derive the area of the latter by difference. From these results there was no difficulty in calculating the weight of protein present in an area equal to that of the window of the counter.

The calculations are greatly simplified if imprints are made of whole leaves, and circular or square pieces of imprint, of a fixed area (25 - 30 cm^2) are taken for measurement of surface area.

Having made all these measurements and calculations we proceed to the determination of surface radioactivity. For this purpose we usually used a BFL-20 end window counter, with a long lead (for in vivo measurements), with a mica window, area 3.14 cm^2, thickness 1.2 - 1.8 mg/cm^2. The advantage of such counters is

that they may be placed on the imprints, for scanning different parts of their surface, and it is convenient to fit metal caps over the window, with apertures of different shapes cut out of them. Ordinary end-window counters may also be used for radioactivity measurements, but if so, it becomes necessary either to cut out pieces of the imprints to use as the sample, or to fix the counter in the "hanging" position, and apply to it the imprint mounted on a movable support. The amount of radiation absorbed by the paper is determined empirically (for S^{35} and C^{14}). For this purpose we measure the activities of very small droplets of radioactive solution, after evaporation to dryness on a mica slide and on filter paper. The surface activity of the imprint multiplied by the absorption coefficient represents the activity of the proteins absorbed on a surface corresponding with the area of the window of the counter.

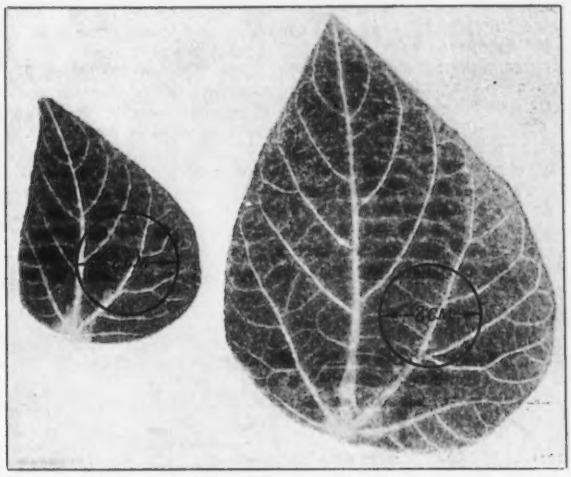

Fig. Imprint of a bean leaf.
The circles outline the areas whose activities were measured by means of an end-window counter.

Knowing the weight of the proteins, we can calculate their Sp. Act. An example of the calculation of the specific radioactivity of the proteins is given below.

Diameter of the sheet of filter paper – 60 mm
Area – 28.26 cm^2
Weight of the paper – 299.7 mg
Weight of the paper + protein – 308.4 mg
Loss of weight of the control sheet of paper – 1.95 mg
Weight of protein in the imprint – 10.65 mg
Weight of the part of the paper not occupied by the imprint – 36.4 mg
Surface area of the imprint – 25.5 cm^2
Area of the window of the counter – 3.14 cm^2
Surface activity of the imprint, per 3.14 cm^2 – 399 imp/min
Weight of proteins contained in 3.14 cm^2 of imprint – 1.3 mg
Activity per 1 mg of protein – 261 imp/min
Absorption of S^{35} radiation by the paper (fast-running chromatographic) – 50.4% (conversion coefficient 2.02).
Specific radioactivity of the protein – 527 imp/min per 1 mg per 3.14 cm^2 of imprint.

If we require, in addition to the Sp. Act. of the proteins, to know the ratio of the total activity of the S^{35} to that of the proteins, or to know the relative proportions of activity present in different fractions, such as, for example, phosphorus-containing products, the activity counts are made before washing and are repeated after each stage of the washing process, having first outlined in pencil the area of the imprint covered by the window of the counter, as is shown in the figure.

The reason for this is that the activity of biochemical processes may vary from part to part of the leaf.

The leaf imprints have the advantage for work with counters of being of uniform thickness and shape. This is of very great importance for ensuring that the geometrical conditions under which the measurements are made are identical, and this gives a higher accuracy of measurement, as compared with measurement of the activity of substances in powder form.

Imprints of labelled leaves may also serve as material for radioautography, since they give the clearest possible picture of the distribution of radioactive substances.

Examples of Experiments, and Some Results Obtained Using The Imprint Method

The object of our first experiment was to ascertain what individual deviations there were in the process of protein synthesis in the leaves of different levels and of different bean plants grown under identical conditions.

The bean plants were grown for a month in hydroponic culture, using the same Hellriegel nutrient medium for all plants. After a month the nutrient medium was renewed (adding one-third of the usual dose of mineral salts), at the same time adding to each vessel 300 μC of S^{35}-labelled sodium sulfate. The reason for this relatively high dose of S^{35} was that bean plants of this age absorb sulfates only very weakly. Leaves were taken from different levels, and from different plants, at various times after exposure to S^{35}, for preparation of imprints, and corresponding leaves were taken at the same time for determination of the ratios of different S^{35}-containing fractions by means of the ordinary analytical methods. The imprints were made on small discs of filter paper (diameter 60 mm), which could accommodate only small leaves from the upper tiers of the plants. Pieces were cut out of the large leaves from the lower levels, for preparing imprints. In all cases we took the upper leaflet of the ternate bean leaf. Imprinting was effected by a blow from a piston, propelled by release of a powerful spring, which was compressed to the same extent on each occasion.

TABLE 1

Specific Radioactivity of the Proteins, and a Number of Other Data for Bean Leaves Labelled With S^{35}

Plant No.	Surface area of the leaf imprint, in cm^2	Protein content of the imprint, in mg	Wt. of protein in 3.14 cm^2 of imprint (area of the window of the counter)	Surface radioactivity, in imp/min per 3.14 cm^2 of surface		% of total S^{35} incorporated into proteins	Radioactivity of 1 mg of proteins, corrected for self-absorption; imp/min per 3.14 cm^2
				total S^{35}	protein S^{35}		
Second tier of leaves							
1	25,5	10,7	1,3	397	339	85,4	522
2	27,6	5,7	0,65	40	24	60,0	74
3	25,2	5,2	0,65	299	131	43,8	404
4	22,1	6,7	0,81	28	10	35,7	24
5	26,7	11,9	1,41	278	163	58,6	230
Third tier of leaves							
1	27,5	8,7	0,99	8 848	7 610	86,1	15 372
2	20,82	4,1	0,62	298	193	64,8	618
3	22,12	3,3	0,47	2 151	627	29,1	2 688
4	18,7	2,1	0,35	303	145	47,8	828
5	16,2	2,3	0,45	940	300	31,9	1 332
Fourth tier of leaves							
1	26,8	10,5	1,1	24 964	18 876	75,9	34 320
2	15,46	4,8	0,87	1 948	1 504	77,1	3 440
3	14,8	6,5	1,38	10 466	4 998	47,5	7 244
4	13,62	3,9	0,90	2 497	1 250	50,2	2 754
5	13,2	3,7	0,89	3 368	1 667	48,3	3 746

Table 1 presents the results obtained for specific radioactivity of the proteins, and for determinations of percentage of total S^{35} present in the proteins 100 hrs after introduction of S^{35}.

The data of Table 1 show that there are quite considerable individual variations in protein synthesis and uptake of sulfur from plant to plant, quite apart from the natural rise in specific activity of the leaf proteins and in the total activity of the sulfur, depending on the level from which the leaves are taken, and hence on their age. These individual differences, which appear to be of physiological importance, are smoothed out when when pooled samples are taken for analysis. The imprintation method not only permits of the discernment of individual peculiarities of leaves, but it also allows us to examine certain new biochemical and physiological correlations. These new possibilities relate first to an analysis of correlations between the rate of supply of the isotope to the leaf, the rate of synthesis of proteins, and their specific activities and such parameters as the surface area of the leaf and its protein content (the weight of the proteins in the imprint is proportional to their concentration in the protoplasm of the corresponding area of the leaf).

In our experiments the area of the leaves was not the same as that of the imprints, since the length of the lower leaves, of the second and third tiers, usually exceeded the diameter of the piston (only a part of these leaves was taken for imprinting). The reason for this was that we were obliged to restrict ourselves to a small surface of impact, as our spring-loaded device was not powerful enough to cope with a larger surface. This limitation could, however, be overcome by designing a more powerful device.

Sampling of whole leaves at appropriate intervals would permit of the correlation of incorporation of isotope into proteins with the intensities of processes of growth and biosynthesis, as manifested by the growth increments of the leaves and the changes in protein content of their cell protoplasm. A more profound physiological interpretation of the radiochemical data might emerge from such correlations.

It should be noted, with reference to the data presented in Table 1, that in the leaves of the third and fourth tiers, maximum specific activity of the proteins is correlated with maximum concentration of proteins in the protoplasm. It is also of interest that the rate of uptake of S^{35} from the nutrient solution by one of the five plants (No. 1) is for some reason many times greater than for the others (plants 1, 2, and 3 were grown in the same vessel).

The ratios of total to protein sulfur found for pooled samples of leaves of a given level were very close to the mean values derived from analyses of imprints of individual leaves taken at the same time and from the same level (about 50% of the total radioactivity of the S^{35} was present in proteins). Direct comparison of the absolute values of the activities of specimens analyzed by the ordinary method with those found for imprints is not possible, because the geometrical conditions of measurement would have to be identical, and this cannot readily be achieved, owing to the different physical states of the specimens in the two cases.

Apart from serving for the determination of specific activity of proteins, the imprint method can also be applied to the evaluation of the relative proportions of different radioactive fractions of leaf constituents. We investigated the possibility of rapidly determining the proportion of total isotopic phosphorus present in nucleic acids.

The plants were grown in sand with Hellriegel's nutrient medium, to which 600 μC of P^{32} was added when filling the pots. This high dosage of P^{32} was taken so as to enable us to follow the variations in the phosphorus-containing fractions over long periods of time. In view of the low initial specific activity of the phosphorus we could see no evidence of any damage due to radiation being suffered by the plants. Leaves were taken for analysis 45 days after sowing the seeds.

The data of Table 2 show that the individual variations in the radioactivity of imprints made from different plants were very small. This is as would be expected, since the total labelling of the plants is determined by the radioactivity of their constituents, rather than by their metabolism. The composition of the plants is less subject to variation than is their metabolism.

In this group of plants we determined total and nucleic acid P^{32} of the leaves, using the imprint method as well as the ordinary method.

The leaves were dried and powdered, and the activity of 200 mg of the powder was measured before and after extraction of soluble fractions and lipids. In view of their high activity, the specimens were placed at a considerable distance from the counter (60 mm) (BFL-25). For this reason, only the ratios found in these experiments (Table 3) can be compared with the data of Table 2, but not the absolute values of the radioactivity of the specimens.

A comparison of the last columns of Tables 2 and 3 shows that the values differ considerably, although the analytical material was taken from one and the same group of plants. We at first thought that these differences were due to adsorption of some of the soluble phosphorus fraction on the paper. However, experiments in which we sprayed unlabelled imprints with labelled phosphate solution showed that, using our washing procedure, not more than 0.2% of the radiophosphorus undergoes adsorption, which could not appreciably affect the results. Further investigations showed that the difference between the two sets of values was a real one, being due to differences in the material taken for assay in the two sets of experiments.

TABLE 2

Surface Radioactivity of Imprints of Bean Leaves Labelled With P^{32}

Serial No. of plant	Surface radioactivity of imprints of leaves, in imp per min per 3.14 cm^2		% of total P^{32} present in nucleic acids
	total P^{32}	nucleic acid P^{32}	
Second tier of leaves			
1	3008	678	22.5
2	2944	578	19,7
3	2828	608	21,5
Third tier			
1	5248	1300	24.8
2	5632	1210	21.5
3	5410	1440	26,4
Fourth tier			
1	7680	2032	26,5
2	7424	1801	24,3
3	7140	2118	29,7

TABLE 3

Ratio of Total to Nucleic Acid P^{32}, as Found by the Usual Method of Assay of Dry Material

Serial No. of plant	Radioactivity in imp/min of 200 mg of dry substance		% of total P^{32} present in nucleic acids
	total P^{32}	nucleic acid P^{32}	
Second tier of leaves			
1	12 640	1844	14,6
2	13 280	1920	14,4
Third tier			
1	23 760	3380	14,2
2	27 120	3496	12,9

In the experiments involving the assay of imprints we determined the ratios of the phosphorus compounds in the cell protoplasm of the leaves, whereas the experiments of Table 3 were performed on whole-leaf powder. Analysis of the leaf residues remaining after expression of the protoplasm into the filter paper showed that the cell walls contained relatively larger amounts of soluble and inorganic forms of phosphorus, as compared with nucleic acid phosphorus, than did the protoplasm. Apart from this, part of the protoplasm squeezed out in making the imprints soaks through to the second and third layer of filter paper. This also affects the relative proportions of the different forms of phosphorus compounds found in the first layer, in the direction of raising the proportion of nucleic acid phosphorus, since nuclear structures and some of the larger plastids are retained in the first layer

of paper, whereas the protoplasm soaking through to the second and third layers is relatively impoverished in nucleic acids. This effect should be borne in mind, although it does not interfere with the results of comparative experiments.

It should be noted that the imprint method permits of the evaluation of the relative proportions of different forms of radiophosphorus-containing compounds separately in such fractions as vacuolar fluid, plasma, and total protoplasm. The procedure for preparation of such differentiated imprints has been described by us in our earlier publications [1-3].

The applicability of the imprint method to comparative quantitative estimation of different forms of phosphorus compounds is shown clearly by the data of Table 4, dealing with the dependence of nucleic acid synthesis in leaves on the level of mineral nutrition.

TABLE 4

Ratio of Total Radiophosphorus to Nucleic Acid Phosphorus 40 Hours After Adding P^{32} to the Culture (under different conditions of mineral nutrition of the plants)

Serial No. of plant	Control			After 10 days of lack of phosphorus		
	Surface radioactivity of imprints, in imp/min per 3.14 cm^2		Nucleic acid P^{32}, as % of total P^{32}	surface radioactivity of imprints, in imp/min per 3.14 cm^2		Nucleic acid P^{32}, as % of total P^{32}
	total P^{32}	nucleic acid P^{32}		total P^{32}	nucleic acid P^{32}	
	Second tier of leaves					
1	5 847	430	7,4	1 866	470	25,3
2	880	176	20,0	961	322	33,5
3	3 805	226	6,0	1 450	328	22,6
4	1 362	194	14,3	1 678	324	19,2
	Third tier of leaves					
1	9 982	684	6,9	1 240	290	22,6
2	2 863	274	9,6	1 329	450	34,1
3	4 378	306	7,0	8 537	1282	15,9
4	1 691	258	15,3	1 636	278	17,05
	Fourth tier of leaves					
1	19 674	2880	14,7	3 281	610	18,6
2	7 878	652	8,3	2 556	660	25,9
3	8 676	684	7,9	10 509	1546	14,7
4	2 547	338	13,3	1 921	546	28,4
	Fifth tier of leaves					
1	27 721	5390	19,4	8 360	1210	14,4
2	6 686	578	8,6	8 141	1316	16,2
3	9 501	1154	12,2	19 509	3168	16,3
4	3 610	449	12,1	5 981	832	13,9

The leaves used for making imprints were taken from experimental plants grown by N. N. Zabolotskii, of the Department of Agrochemistry, who was investigating the effect of different conditions of mineral nutrition on incorporation of P^{32} into individual nucleotides entering into the composition of nucleic acids. In this research, the bean plants were grown in hydroponic culture, with all the necessary mineral nutrients. Some of the plants were transferred to a phosphorus-deficient medium 10 days before introduction of P^{32} into the solutions. The phosphate content of the medium was made up to that of the control group at the same time that P^{32} was added to all the culture media (450 μC), so that the specific activity of phosphorus was the same in both series. Leaves were taken for assay 40 hours after introduction of P^{32}.

It may be concluded from the data of Table 4 that incorporation of P^{32} into nucleic acids takes place more actively after phosphorus deprivation; this appears to be related to the smaller dilution of P^{32} entering

the cells, since the concentration of soluble phosphorus compounds in the cytoplasm is considerably lowered under conditions of phosphorus lack. Quite considerable individual variations both in the rate of uptake of phosphorus and in the rate of synthesis of nucleic acids are also evident. The differences between the control and the experimental groups of plants are less marked in the upper, young leaves, which had evidently not suffered phosphorus deprivation, availing themselves of phosphorus from the lower levels. Considerable individual variations were also found here in measuring the activity of newly opened leaves, showing that they cannot have been produced by the method applied by us.

We believe that the experimental material and the concepts presented in this paper show that the procedure depending on the preparation of imprints of leaves on filter paper is of value for the rapid estimation of a number of substances present in plant material. It is probable that the procedures described by us for leaves could also be applied to large slices of animal tissues.

SUMMARY

A new procedure is described, serving for the rapid assay of the specific radioactivity of leaf proteins, and for the estimation of the relative proportions of various fractions of radioactive substances present in leaves.

The leaf is placed on a weighed sheet of filter paper, and struck by a spring-loaded piston; this squeezes out fluid, giving an imprint of the leaf. The imprint is treated with 10% trichloroacetic acid to fix proteins, water-soluble substances and lipids are removed by washing, and the sheet is dried and reweighed, giving the protein content of the imprint, which is calculated per unit area. The specific radioactivity of the protein is calculated from the results of measurements of the surface radioactivity of the imprints. The relative proportions of various radioactive fractions are evaluated without weighing the sheets, from measurements of surface activity of the imprints at various stages of their selective extraction with appropriate solvents.

The method is quick and simple, and is applicable to mass serial determinations of specific activity of labelled substances, and of the relative proportions of such substances in the protoplasm of the leaf cells.

LITERATURE CITED

[1] Zh. A. Medvedev, Izv. Timiriazevskoi J. s. kh. Akad. 3, 186 (1957).

[2] Zh. A. Medvedev, Priroda 8, 90 (1957).

[3] Zh. A. Medvedev, Botan. Zhur. 43, 61 (1958).

[4] Zh. A. Medvedev, Doklady Akad. Nauk SSSR 117, 860 (1957).

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CHEMICAL COMPOSITION OF THE VARIOUS REGIONS OF THE LUMBAR ENLARGEMENT OF THE SPINAL CORD

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On the basis of extensive experimental data, Palladini [1] suggested that the various parts of the nervous system having different anatomical and histological structure and serving different functions differ from each other in chemical composition. Valuable data have been obtained in recent years from the study of the "chemical topography" of the cerebral cortex [2-5]. The spinal cord presents great interest in this respect.

Clear-cut functional and histological differences between the structures of the spinal cord permit us to assume the existence of differences in their chemical composition.

Data presented in this paper were obtained by us during an investigation on the content of dry matter, ash, total and residual nitrogen, and the distribution of trace elements in various regions of the lumbar enlargement of the spinal cord, namely: the whole section of the lumbar enlargement, its white matter, grey matter of the posterior and anterior horns and its posterior and anterior roots.

METHODS

Experiments were carried out on the lumbar enlargement of the spinal cord of the cow. The experimental material was obtained from Dnepropetrovsk meat packing plant, 7-12 min after the animal was killed by severance of its carotid artery. The cord was delivered to the laboratory and divided into various parts on ice. The selected tissue from various regions of the lumbar enlargement was separately cut into small fragments with scissors and ground in an ice-cold agate motar. Three hundred mg of tissue was taken from each region for the determination of dry residue, ash and residual nitrogen, and 100 mg was taken for the determination of total nitrogen. For the determination of residual nitrogen, the protein was precipitated with 3 ml of 20% TCA and 1 ml of the filtrate was taken for ashing. Total and residual nitrogen was determined by the Kjeldahl micromethod.

Dry residue was determined in a platinum crucible at 105°. The dry residue was incinerated in the same crucible in a muffle furnace at 400-420° for 8 hours. The ash, so obtained, was examined for trace elements by spectral emission analysis. Specimens for ashing were taken separately from each region of the lumbar enlargement. Spectral emission analysis of the ash was carried out in the Department of Optics of the Dnepropetrovsk University. The survey was carried out in a ISP-22 spectrograph at 220 volts and 7 amperes; exposure time for the samples and the standards was 3 min (according to Voinar) and for iron, 20 seconds. The sample (20 mg) was burnt in front of the slit (width 0.005 mm) of the spectrograph between electrodes of carbon, purified by the method of Rusanov [6]. The source of excitation was the Sventitski activated arc, using generator PS-39. Spectral photoplates, type II, with light sensitivity of 16 GOST units were used; development was carried out in a standard developer for 4 min. The blackening of the analytical line was measured with a MF-2 microphotometer. The concentration of the trace elements was calculated graphically. The logarithm of the concentration was plotted as the abscissa and the difference between the blackening of the analytical line and the background was plotted as the ordinate. The concentration of each trace element was determined from the graph from the difference between the blackening of the analytical line and that of the background.

EXPERIMENTAL RESULTS AND DISCUSSION

Data regarding the content of dry residue in various regions of the lumbar enlargement of the cow spinal cord are presented in Table 1.

TABLE 1
The Content of Dry Residue in Various Regions of the Lumbar Enlargement of Spinal Cord
(data from 12 experiments, in %)

Whole section	White matter	Grey Matter			Roots	
		anterior horn	posterior horn	as a whole	anterior	posterior
Average						
33,21	34,99	24,59	25,22	25,51	30,07	32,01
Minimum						
30,52	33,99	23,21	23,28	23,88	28,44	29,95
Maximum						
34,90	36,52	25,79	26,41	26,99	31,73	34,27

The data of Table 1 show that the white matter of the lumbar enlargement of the spinal cord contains the greatest quantity of dry matter. It is followed by the whole section of the lumbar enlargement (without roots), posterior roots, anterior roots, grey matter as a whole (without separation into horns), gray matter of the posterior horn and lastly, grey matter of the anterior horn, which contains the least amount of dry residue. Our data on the content of dry residue in roots and in grey matter of the spinal cord agree with the data of Palladini[1]. In the grey matter, the content of dry matter is less in the anterior horn than in the posterior horn.

Results of the investigations on the ash content of tissues of various regions of the lumbar enlargement of the spinal cord are presented in Table 2.

TABLE 2
The Ash Content of the Various Regions of the Lumbar Enlargement of Spinal Cord
(data from 12 experiments, in %)

Whole section		White matter		Anterior horn		Posterior horn		Grey matter		Anterior root		Posterior root	
in fresh tissue	in dry tissue	in fresh tissue	in dry tissue	in fresh tissue	in dry residue	in fresh tissue	in dry residue	in fresh tissue	in dry residue	in fresh tissue	in dry residue	in fresh tissue	in dry residue
Average													
3,44	10,59	4,20	11,64	2,00	7,72	2,37	9,19	2,33	9,51	2,70	10,76	3,22	11,36
Minimum													
3,04	8,14	2,98	8,48	1,59	6,52	1,74	7,18	2,10	7,48	2,30	8,63	2,86	9,52
Maximum													
3,94	12,43	4,86	13,48	2,34	10,00	2,96	10,52	2,74	10,94	2,99	13,09	3,93	12,64

Analysis of the figures for different trace elements presented in Table 3 indicates, that in comparison with other trace elements, copper is present in the largest amount in the spinal cord. Its magnitude is approximately 10^{-2}% of ash in white matter and approximately 10^{-3} % of ash in the other parts. Aluminum comes

next to copper in magnitude of content in the lumbar enlargement of spinal cord. It is distributed unevenly in the regions investigated. The highest concentration of aluminum (approximately 10^{-3}% of ash) is observed in the white matter and in the grey matter of the anterior horn; approximately half of this concentration occurs in the anterior root and in the tissue of the whole section of the cord; it is approximately 10^{-4}% of ash in the posterior root. Silicon is concentrated predominantly in the posterior and anterior roots and in the white matter of the cord in a concentration of 10^{-3}% of ash. This is apparently related to the relatively large content of supporting tissues in these regions.

TABLE 3

The Content of Trace Elements in the Lumbar Enlargement of Spinal Cord
(as % of ash by spectral emission analysis)

Region of the Lumbar Expansion of Spinal Cord	Cu	Al	Silicon	Titanium	Chromium	Mn	Ni	Lead	Silver
Whole Section	0,0055	0,0031	0,001	0,002	0,001	0,0005	0,001	0,0003	traces
White Matter	0,022	0,0075	0,004	0,002	0,001	0,001	0,001	0,0019	traces
Grey Matter	0,003	0,0066	0,002	0,003	0,001	0,0015	0,0015	0,0004	traces
Grey Matter of Anterior Horn	0,002	0,0072	0,003	0,002	0,0005	0,0005	Her	0,0002	traces
Grey Matter of Posterior Horn	0,004	0,0057	0,001	0,002	0,0014	0,0025	0,001	0,0006	traces
Anterior Root	0,003	0,0038	0,005	0,003	0,001	0,0005	0,001	0,0009	traces
Posterior Root	0,001	0,0006	0,006	0,003	0,001	0,0003	Her	0,001	traces

Titanium and chromium are distributed evenly in all of the investigated regions of the spinal cord, in a concentration of about 10^{-3}% of ash, with the exception of the grey matter of the anterior horn, where the content of chromium is somewhat less. Manganese is found in all of the investigated regions of the lumbar enlargement of spinal cord in a concentration of about 10^{-3}% of ash. The manganese content of the grey matter of the posterior horn is approximately 5 times as great as that of the grey matter of the anterior horn; the manganese content of the roots is about half that of the white matter. Nickel is distributed unevenly. It was not detected in the grey matter of the anterior horn and in the tissues of the posterior roots. Nickel content of the rest of the investigated regions was of the order of 10^{-3}% of ash. Lead is distributed evenly in all the investigated regions of the lumbar enlargement of the spinal cord in a concentration of about 10^{-4}% of ash, with the exception of the white matter and the root tissues where its content approaches 10^{-3}% of ash.

Of the trace elements investigated, silver occurred in the least concentration in the lumbar enlargement of spinal cord. Traces of silver were found in all regions of the lumbar enlargement of spinal cord and it attained a concentration of about 10^{-3}% of ash in the grey matter of the posterior horn.

The trace elements (in % of ash) may be arranged in the following series in descending order of their concentration in the lumbar enlargement of spinal cord and its roots:

White matter	0,0404
Grey matter (without subdivision into parts)	0,0190
Grey matter of posterior horn	0,0183
Tissue of anterior root	0,0182
Grey matter of anterior horn	0,0154
Tissue of the whole section of the lumbar enlargement	0,0144
Tissue of posterior root	0,0120

The data regarding the quantitative distribution of trace elements in the lumbar enlargement of spinal cord, cited here, confirm the suggestion of Palladini that the cord tracts with different morphological structure and physiological function possess different chemical composition.

The elucidation of the quantitative distribution of total and residual nitrogen as indices of protein content is important in the study of the chemical topography of the cord. The results of our investigations on the

content of total and residual nitrogen in various regions of the lumbar enlargement of spinal cord are presented in Table 4. These data indicate that the investigated regions of the cord contain different amounts of total nitrogen. The highest amount of total nitrogen is contained in the grey matter of the anterior horn and a slightly smaller amount is found in the grey matter of the posterior horn. A minimal quantity of nitrogen is found in the white matter. The roots occupy an intermediate position; however, total nitrogen is greater in the anterior roots than in the posterior roots. Our data on the content of total nitrogen in the roots agree with the data of Palladini [1].

TABLE 4

Total and Residual Nitrogen Content of Various Regions of the Lumbar Enlargement of Spinal Cord

(data from 8 experiments, in % of fresh tissue)

White matter		Grey matter				Roots			
		anterior horn		posterior horn		anterior		posterior	
total nitrogen	residual nitrogen	total nitrogen	residual nitrogen	total nitrogen	residual nitrogen	total nitrogen	residual nitrogen	total nitrogen	residual nitrogen
Average									
1,85	0,19	2,35	0,27	2,29	0,24	2,09	0,18	1,99	0,18
Minimum									
1,62	0,09	1,93	0,11	1,90	0,10	1,76	0,08	1,68	0,09
Maximum									
2,38	0,30	2,76	0,52	2,72	0,36	2,53	0,24	2,48	0,29

On comparison of the data of Tables 1 and 4, an inverse relationship between the content of dry matter and total nitrogen in the investigated regions of the lumbar enlargement of spinal cord is easily noticed. The regions of the cord that contain the highest amount of dry residue also contain the least amount of total nitrogen and vice versa. This relationship is not violated if the magnitude of residual nitrogen is deducted from the total nitrogen in order to calculate, approximately, the protein content. Lipoid nitrogen was not investigated.

Since an inverse relationship exists in the cord between the dry residue and water content, one may postulate that the regions of the lumbar enlargement which contain comparatively larger amounts of protein also contain higher amounts of water. If one accepts the view of Palladini that the regions of the cord containing the highest amounts of protein and water carry out the most complicated functions, one can assume that the most intensive metabolic and physiological processes take place in the grey matter of the anterior horn. In this respect, the anterior horn is followed by the grey matter of the posterior horn, tissue of the anterior roots, posterior roots, and lastly by the white matter.

SUMMARY

Various regions of the lumbar enlargement of cow spinal cord contain different amounts of dry residue, ash, total nitrogen and trace elements.

According to the content of dry matter and ash as percentage of fresh tissue, the regions of the lumbar enlargement of spinal cord studied may be arranged in the following descending order: white matter, posterior roots, anterior roots, grey matter of the posterior horn, and the grey matter of the anterior horn. The investigated regions form a reverse order on arranging them according to their content of total nitrogen.

From an analysis of the data obtained, one can assume that the most intensive metabolic and physiological processes take place in the grey matter of the anterior horn which contains more protein and water than any other region investigated. In this respect, the anterior horn is followed by grey matter of the posterior horn, tissue of the anterior root, posterior root and finally, the white matter. This conclusion is in agreement without previous data, obtained during an investigation of the functional characteristics of the various links of the reflex arc of spinal cord during anesthesia [7].

LITERATURE CITED

[1] A. V. Palladini, Ukr. Biokhim. Zhur. 7, 5 (1935); 9, 170 (1936); Fiziol. Zhur. 21, 493 (1936).

[2] A. V. Palladini and B. I. Khaikina, Advances in Biological Chemistry, Vol. II, p. 27, Medgiz, 1954.*

[3] I. L. Ben'kovich, Protein Metabolism of the Brain, Sev.-oset. Giz. Dzaudzhikau, 1946.*

[4] A. O. Voinar and A. K. Rusanov, Biokhimiia 14, 102 (1949).

[5] S. I. Dorfman and S. A. Shipitsyn, Biokhimiia 20, 136 (1955).

[6] A. K. Rusanov, Spectral Analysis of Ores and Minerals, Gostekhizdat, 1948 .*

[7] A. D. Reva, Reports of Proceedings of the Eighth All-Union Congress of Physiology, Biochemistry and Pharmacology, p. 502 Medgiz, 1955.*

[8] S. D. Balakhovskii and I. S. Balakhovskii, Methods of Chemical Analysis of Blood, Medgiz, 1953. *

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TOBACCO ALKALOIDS AND NITROGEN METABOLISM

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The synthetic processes taking place in the root system are now recognized as being of particular importance for the vital activaties of plants [1, 2]. This has been established by numerous researches dealing with plant grafts. Thus, nicotine has been shown to be formed in the roots of the tobacco plant, from whence it is carried up in the sap, and accumulates in the aerial parts of the plant [3]. Fresh evidence of this phenomenon has been provided by experiments involving the use of radioactive isotopes [4].

Emerging from the hypothesis that nicotine synthesis takes place when nitrogenous nutrients enter the root system, and that it is concerned in general nitrogen metabolism, we set ourselves the task of elucidating the connections between nicotine synthesis, content of amino acids, and protein synthesis in the root system. This approach was based on published data indicating that certain amino acids might serve as precursors for the biosynthesis of alkaloids.

We examined the sap of tobacco plants, as being the immediate product of the vital activities of the root system, and which rises to the aerial parts of the plant, where it participates, together with other products, in the metabolism of the plant.

Sabinin and his collaborators have performed numerous and fruitful researches on plant sap, in which they studied the uptake of ions from the outside medium, and the transformations undergone in the root system of plants by certain inorganic materials (nitrogen, phosphorus); they also established the relation of this process to the basic physiological functions of plants, such as, for example, respiration [5]. Kursanov and his collaborators showed that certain organic acids, in particular malic, fumaric, citric, succinic, pyruvic, and oxoglutaric acids, were present in the roots and sap of pumpkins. In addition, the amino acids alanine, glycine, aspartic acid, glutamic acid, cystine, lysine, and threonine were found [6, 8]. Eleven amino acids were found in the sap ascending from the roots of sterile cultures of corn plants [7].

Earlier studies of tobacco plant sap had as their object to find out where nicotine synthesis took place [9-11].

For our researches we took Nicotiana tabacum, Nicotiana glutinosa, and Nicotiana glauca plants, which contained the chief alkaloids of tobacco – nicotine, nornicotine, and anabasine.

The plants were grown in greenhouses, under the ordinary conditions. At the time of sampling, the vegetative parts of the plants were well developed; the plants were 4-5 months old. The stem was cut immediately above its emergence from the root, and a rubber tube, previously treated with alcohol and wrapped in sterile linter, was fitted over the stump. The sap was collected, and at once taken for analysis. It was quite clear, and no evidence of growth of microorganisms in it could be seen. Not all of the plants yielded sap; some produced none at all, evidently because of some structural or physiological peculiarities. Alkaloids were determined in the root sap according to Pfeil [12], by steam distillation followed by precipitation with picric acid as dipicrates.

We applied one-way ascending paper chromatography to the determination of free amino acids in the root sap of the three Nicotiana species.

The sap was applied as streaks to the chromatography paper, without any preliminary treatment, as recommended by Hiller et al. [13]. We used Leningrad Factory slow paper, previously treated with the appropriate buffer solution. The solvent was passed perpendicular to the direction of the fibers of the paper, which was determined by a water-drop test. We used the following solvent systems for the separation of the free amino acids:

1. Phenol saturated with phosphate buffer, pH 12 (0.067 M). The paper was treated with the same buffer. According to MacFarren [14] this solvent gives good resolution of mixtures of aspartic and glutamic acids, serine, and glycine. The solvent was run twice.

2. o-Cresol saturated with phosphate buffer, pH 6.2 (0.067 M). The paper was previously treated with the same buffer. This solvent is suitable for the separation of alanine, tyrosine, valine, methionine, leucine, and phenylalanine (MacFarren [14]). The chromatogram was run for 48 hours.

3. 4:1:1 n-Butanol – acetic acid – water. This solvent was used for separation of cystine, lysine, arginine, and proline.

Lysine separated out after the solvent had been run five times.

We treated the chromatograms with 2% ninhydrin in acetone, containing 1% of acetic acid. The strips were passed through the ninhydrin solution in a direction opposite to that of the flow of solvent [15]. The colors were then developed by heating the chromatograms at 60° for 30 min. The colored spots were cut out of the paper, and extracted. For extraction, with simultaneous fixation of the coloration, we used a 4% solution of $CaCl_2$ in 40% methanol [16]; elution time 2 hours, with shaking. The optical density of the colored solutions was read in a SF-4 spectrophotometer, at 510 mμ. The amino acid content of the extracts was derived by comparison with standard solutions. In all cases we applied alternate streaks of test and reference solutions to the same sheet of paper. We prepared calibration curves for proline, arginine, and histidine.

TABLE 1
Characteristics of the Root Sap of Various Tobacco Plants

Species	pH	Alkaloid content, mg/ml	M. p. of dipicrates of the alkaloids	Alkaloid present
N. tabacum	5,35	0,12	218	Nicotine
N. glutinosa	5,95	0,09	218	Nicotine
N. glauca	5,95	0,10	203	Anabasine

Apart from ninhydrin, we used a number of other specific reagents for color development. Proline, in particular, was identified by means of its specific reaction with isatin. The reagent was 0.4% isatin in n-butanol containing 4% of glacial acetic acid [14]. This gives a deep blue color on a yellow background. The intensity of the coloration was measured directly on the paper [18], with the aid of a Shipalov photoelectric densitometer, with automatic registration [19]. The complex breaks down when elution is attempted. Arginine was determined by the method of Rosenberg et al. [20], which depends on the formation of a reddish violet complex of arginine, α-naphthol, and diacetyl in alkaline solution. The optical density of the solution was measured at 535 mμ in a SF-4 spectrophotometer. Histidine was determined by the method of Fraenkel-Conrat and Singer [21] (reaction with diazotized sulfanilic acid).

The amino acid composition was expressed as percentage of total nitrogen present in amino acids per 1 ml of sap. Total nitrogen was determined by a micro-Kjeldahl method.

The amount of sap collected from individual plants (Table 1) varied from 0.2 to 0.5 ml/hr. Its pH was 5.5, being the same as that of the contents of the leaf cells. The alkaloid content of the sap amounted to 0.1 mg/ml. The m. p. of the dipicrates coincided with those of nicotine and anabasine. N. glutinosa forms nicotine in the root system, and this is demethylated in the leaves, to yield nornicotine, which usually accumulates in this species. Thus, analysis of the root sap of our plants shows the presence of the alkaloids characteristic of these plants, and this finding is in full agreement with the data obtained earlier for grafts of these species

on plants not producing alkaloids. Biosynthesis of nicotine takes place in the root systems of tobacco plants, whereas anabasine is formed in both the roots and the leaves of N. glauca.

TABLE 2

Content of Free Amino Acids in Tobacco Plant Root Sap

Amino acid	N. tabacum (total N - 0.175 mg/ml) amino acids		N. glutinosa (total N = 0.152 mg/ml) amino acids		N. glauca (total N- 0.113 mg/ml) amino acids	
	μg/ml	as % of total N	μg/ml	as % of total N	μg/ml	as % of total N
Glycine	—	—	24,7	3,02	—	—
Alanine	90	8	120	11,8	101	14,1
Valine	9	0,57	15	0,65	5,9	0.62
Leucine	26	1,7	21	1,31	16,7	1,47
Serine	traces	traces	—	—	—	—
Threonine	—	—	49	3,94	—	—
Methionine	3,7	0,2	5	0.3	9,5	0,8
Phenylalanine	8,5	0,4	8,5	0,46	17	1,23
Tyrosine	16,5	0,57	21	1,31	28,2	1,76
Aspartic acid	2	0,1	10,2	0,65	7	0,62
Glutamic acid	15,7	0,85	11,3	0,65	—	—
Arginine	36,5	4,6	38,6	5,92	24,3	5,1
Proline	26,2	1,7	19,1	1,31	traces	traces
Histidine	12,5	1,7	5,2	0,65	12	2,04
Tryptophan	9.6	0,57	41	3,2	—	—

The analytical results for the content of amino acids in the root sap of tobacco plants, presented in Table 2, show that the identity and amounts of the individual amino acids are fairly constant, and are characteristic of a given species. In all we detected 15 amino acids, which is evidence of the capability of the root system of tobacco plants of synthesizing practically the whole range of amino acids required for normal nitrogen metabolism. Our plants showed certain differences with respect to the synthesis of individual amino acids, which are evidently connected with their power of synthesizing one or another alkaloid. Thus, the sap of N. glutinosa contains glycine and threonine, which are absent from that of N. glauca and N. tabacum. N. glauca sap does not contain glutamic acid, and proline is present in traces only. The root sap of N. glauca differs markedly from that of other species with respect to these amino acids. Alanine is the most abundant amino acid of the sap of all three species, pointing to the high activity of amination processes in the root system [22]. However, glutamine may have moved together with alanine, although we were not able to detect it. The amino acids of root sap may be arranged in the following diminishing order of abundance: alanine, arginine, proline, leucine, histidine, valine, tyrosine, phenylalanine, tryptophan, aspartic acid.

Of particular interest are those amino acids which may be possible biosynthetic precursors of the alkaloids.

Researches on the biosynthesis of nicotine, involving the use of C^{14}-labelled amino acids, have shown that ornithine contributes to the formation of the pyrrolidine ring of nicotine [23, 24], and methionine and glycine provide the methyl groups of nicotine [25, 26]; lysine enters into the piperidine ring of anabasine [27]. Thus the theoretical indications pointing to amino acids as possible precursors of alkaloids have been fully confirmed [28]. The presence of C^{14} in the molecules of these alkaloids is evidence that amino acids enter into the metabolic pathways leading to the biosynthesis of nicotine. Nicotine, which is a specific metabolite of the tobacco plant, is formed in the root by a series of biochemical reactions, which are known with certainty to involve the participation of amino acids. No similar data are available for products of carbohydrate metabolism, which would represent the starting point for the construction of the complex nicotine skeleton. There is only Emde's hypothesis that hexoses and trioses take part in this synthesis [29].

The amino acids present in the root sap are fully able to satisfy the requirements for the biosynthesis of nicotine; the non-nitrogenous substrates are also evidently available.

Our work on the root sap of tobacco plants has shown that the roots are able to synthesize a number of complex organic compounds, including nearly the complete set of amino acids, and the specific product of the metabolism of the root system— nicotine. There is evidence that certain of the amino acids are concerned in the biosynthesis of nicotine.

It may be concluded from our experimental results that the root system of tobacco plants contains all the necessary precursors for the biosynthesis of nicotine. The next stage of these researches will be the study of the biosynthesis of nicotine in model systems containing its precursors.

The presence of di- and tricarboxylic acids of the Krebs' cycle, together with the amino acids identified by us, is evidence in support of the existence of a connection between carbohydrate and nitrogen metabolism in that part of the root system in which synthesis of nicotine takes place. It is on this that we base our hypothesis that the pathway of biosynthesis of nicotine may involve products of both carbohydrate and nitrogen metabolism, some of which have been identified and shown to be present in the root system of the plants.

Although the mechanism of biosynthesis of tobacco alkaloids is not yet fully known, we believe that they take part in the general nitrogen metabolism of the plant.

SUMMARY

The root sap of various Nicotiana species has been shown to contain nicotine or anabasine, as well as a number of amino acids; there are certain specific differences in the nature and the amounts of the amino acids found, which appear to be related to the metabolic peculiarities of the root systems of the given species.

The range of amino acids found includes arginine, glutamic acid, glycine, methionine, ornithine, and lysine, which have been mentioned in the literature as possible precursors of nicotine and anabasine.

In view of the presence in the root systems of the plants of organic acids, such as pyruvic, oxaloacetic, and α-oxoglutaric acids, as necessary metabolites for the synthesis of amino acids and amides, we may regard the root system as an organ capable of performing a number of different functions, including the synthesis of nitrogen compounds which are conveyed with the sap to the aerial parts of the plant.

LITERATURE CITED

[1] D. A. Sabinin, IX Timiriazev Lectures, (Izd. AN SSSR, Moscow, 1949).*

[2] K. Mothes, Abhandl. Deutsch. Akad. Wschaft. Berlin 6, 1 (1956).

[3] K. Mothes, Ann. Rev. Plant Physiol. 6, 393 (1955).

[4] G. S. Il'in, Fiziol. rastenii 2, 573 (1955).

[5] D. A. Sabinin, "Physiological Principles of Plant Nutrition," (Izd. AN SSSR, Moscow, 1955).*

[6] A. L. Kursanov, O. F. Tueva, and A. G. Vereshchagin, Fiziol. rastenii 1, 12 (1954).

[7] I. I. Kolosov and S. F. Ukhina, Fiziol. rastenii 1, 37 (1954).

[8] A. L. Kursanov and O. G. Kulaeva, Fiziol. rastenii 4, 322 (1957).

[9] K. Mothes and K. Hieke, Naturwissenschaften 31, 17 (1943).

[10] H. Schmid, Ber. Schweiz. Bot. Ges. 58, 5 (1948).

[11] G. Reuter, Kulturpflanze 1, 260 (1956).

[12] A. A. Shmuk, "Chemistry of Tobacco and Makhorka," (Izd. Pishcheprom, Moscow, 1938).*

[13] E. Hiller, F. Zinnert, and G. Frese, Biochem. Z. 323, 245 (1952).

[14] E. F. McFarren, Anal. Chem. 23, 168 (1951).

[15] T. Fischer, Biochem. Z. 324, 544 (1953).

* In Russian.

[16] S. Lissitzky, G. Césaire, and R. Massonet, Bull. Soc. Chim. biol. 36, 655 (1954).

[17] B. Keil, Khimia (Czechoslovakia) 5, 1006 (1954).

[18] R. Achee, C. Fromageot, and M. Jutisz, Biochim. biophys. acta 5, 81 (1950).

[19] M. S. Shipalov, All-Union Inst. of Sci. and Tech. Information, (Izd. Gostekhnika SSSR, Moscow, 1958). *

[20] H. Rosenberg, A. Ennor, and J. Marrison, Biochem. J. 63, 153 (1956).

[21] H. Fraenkel-Conrat and R. Singer, Arch. Biochem. Biophys. 65, 296 (1956).

[22] V. Kretovich, A. Bundel' and K. Aseeva, Doklady Akad. Nauk SSSR 80, 225 (1951).

[23] E. Leete, Chem. Industry 19, 537 (1955).

[24] L. Dewey, R. Byerrum, and C. Ball, Biochim. biophys. acta 18, 141 (1955).

[25] L. Dewey, R. Byerrum, and C. Ball, J. Amer. Chem. Soc. 76, 3997 (1954).

[26] R. Byerrum, H. Robert, and C. Ball, J. Biol. Chem. 210, 645 (1954).

[27] E. Leete, J. Amer. Chem. Soc. 78, 3520 (1956).

[28] K. Shepf, Uspekhii Khim. 9, 1015 (1940).

[29] N. Emde, Naturwissenschaften 36, 699 (1929).

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* In Russian.

INTERRELATIONS BETWEEN CARBOHYDRATE AND NITROGEN METABOLISM OF THE OAK SILKWORM PUPA DURING METAMORPHOSIS

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We showed in an earlier communication [1] that glycogen synthesis takes place during the development of the oak silkworm pupa. We found that glycogen synthesis takes place from the 4th to the 8th, and sometimes to the 12th, day of development. Only breakdown of glycogen is found thereafter, down to emergence of the imago.

It is known that the initial stages of metamorphosis involve histolytic processes, and that this phase is succeeded during the second third of the period of development by new formation of imaginal tissues, which is associated with intensified synthesis of proteins.

It was thought to be of considerable interest to find what substances are used for the synthesis of protein, and to ascertain how the glycogenolytic process is related to that of protein synthesis during the second third of the period of metamorphosis. Sisakian and Kuvaeva [2] advanced the view that protein synthesis and polysaccharide breakdown were interrelated during this phase, in the sense that the energy derived from the breakdown of polysaccharides was utilized in the process of protein synthesis.

In order to elucidate the question of the connection between nitrogen and carbohydrate metabolism we made parallel determinations of the glycogen content of developing pupae and of their total protein and amino group nitrogen.

EXPERIMENTAL METHODS

Glycogen was isolated from tissues by Ostern's trichloroacetic acid (TCA) method [3], with the difference that the number of extractions of glycogen was raised to five. Glycogen was precipitated with alcohol, washed with 60% alcohol, dried on the water bath, dissolved in water, and hydrolyzed with 2.2% HCl. The resulting glucose was determined according to Issekutz. We took about 0.5 g of air-dry tissue for the estimation.

Total nitrogen was determined by a semimicro-Kjeldahl method. For this purpose we took about 0.2 g of air-dry tissue.

Protein nitrogen was determined by the Kjeldahl method, in the residue remaining after grinding up the air-dry tissue with quartz sand and phosphomolybdic acid (PMA) solution. The suspension was filtered through two thicknesses of filter paper on a Buchner funnel, and the residue was again treated with PMA and again collected on the filter. This extraction was repeated five times in all, after which the final residue was washed a number of times with PMA, on the filter. We took 0.5 g of air-dry tissue for this analysis. We used PMA instead of the more commonly employed TCA in view of the finding of Gorbach, Dedic and Philippi [4] that PMA gives more complete precipitation of protein than does TCA. Apart from this, PMA does not precipitate amino acids together with proteins.

Amino group nitrogen was determined by Van Slyke's method in the filtrates from protein precipitation. Owing to their relatively low amino acid content, the filtrates from two parallel specimens were pooled, made neutral with solid sodium carbonate, and evaporated down to small volume, in a Claisen flask at not more than 40°, and under reduced pressure. The residue was transferred quantitatively to a 25 ml measuring flask, which was filled to the mark, and aliquots were taken for analysis.

Two lots of pupae were taken for incubation: 45 females and 60 males. The temperature of incubation was 20-22°. We took 5 females or 6 males for each test sample. The pupae were fixed in ten times their weight of boiling alcohol, for 30 min , after which they were left for a few days in alcohol, for their more complete dehydration. The alcohol was then evaporated off in vacuum, and the air-dry pupae were powdered, and stored in this form. All the analyses were performed on air-dry material. We determined the percentage content of dry matter in a separate portion of material, so as to enable us to relate the analytical results to dry weight.

RESULTS

Analytical samples were taken on the 1st, 4th, 8th, 9th, 13th, 17th, and 20th days of incubation, and on emergence. The analytical results are presented in Table 1.

TABLE 1

Total, Protein and Amino Group Nitrogen and Glycogen Contents of Oak Silkworm Pupae at Various Stages of Development

Day of development	Sex of pupa	Total nitrogen, as% of dry wt	Protein nitrogen		Amino group nitrogen		Glycogen, as % of dry wt
			as % of dry weight	as % of total nitrogen	as % of dry weight	as % of total nitrogen	
1	♀	9,43	7,44	78,9	0,933	9,89	6,23
	♂	8,38	6,47	77,2	0,852	10,17	4,06
4	♀	9,47	8,17	86,3	0,623	6,58	6,69
	♂	8,45	6,15	72,8	0,788	9,32	4,09
9	♀	9,93	7,73	77,8	0,678	6,83	6,38
	♂	8,67	6,71	77,4	0,624	7,20	4,15
13	♀	9,91	8,36	84,4	0,522	5,26	6,80
	♂	9,15	7,08	77,4	0,645	7,05	3,09
17	♀	10,39	9,21	88,6	0,467	4,49	4,92
	♂	9,13	7,75	84,9	0,432	4,73	1,39
20	♀	11,45	10,08	88,0	0,392	3,42	1,43
	♂	9,75	8,73	89,5	0,329	3,37	0,14
Moths	♀	10,99	10,15	82,4	0,259	2,36	2,07
	♂	8,11	7,24	89,3	0,176	2,17	0,08

An examination of the results shows that the total and protein nitrogen contents and the glycogen content of females are higher at all stages of development than of males, as has previously been pointed out by a number of workers. Only on the first day of development is the amino group nitrogen content of females higher than of males, the values being approximately equal thereafter. If, however, amino group nitrogen is expressed as percent of total nitrogen we find that, except on the 20th, final day of development, the values are consistently higher for males than for females. Very similar values are found for the protein nitrogen contents of males and females, expressed as percent of total nitrogen (77.2 and 78.9%, respectively, on the first day); the protein nitrogen content rises in both sexes as development proceeds. We found that protein synthesis proceeded intensively. In females, the protein nitrogen content rose from an initial value of 7.44% to one of 10.08% of the dry weight on the 20th day for females, and from 6.47 to 8.73% for males.

Protein nitrogen expressed as percent of total nitrogen rose by 9.1% on the 20th day for females, and by 12.3% for males; expressed as percentages of the initial values the corresponding figures are 13% for females and about 16% for males.

Comparison of the fall in amino group nitrogen with the rise in protein nitrogen shows clearly that the latter cannot have been achieved at the cost of the former. This suggests that protein synthesis taking place duirng pupal development is effected not only from free amino acids present in the pupal tissues, but also from some other, as yet unidentified nitrogen compound.

The variations in glycogen content shown in Table 1 are of interest. As was pointed out in our previous communication [1], the glycogen content of the males differs from that of females, and falls abruptly in both between the 9th and the 13th day of development, after a slight rise toward the 9th day.

This abrupt fall in glycogen content has long been known to take place in the mulberry silkworm and has been regarded as being due to the need to cover energy requirements of the pupa during the second third of its development period.

It is, however, possible that in the given case protein synthesis and glycogenolysis are linked processes. Not only would the energy of breakdown of polysaccharides be utilized for protein synthesis, as has been suggested by Sisakian and Kuvaeva [2], but the products of degradation of glycogen may also serve as substrates for protein synthesis.

Demianovskii and Gal'tsova [5] have reported that amination of pyruvic and oxaloacetic acid takes place in the fat body of the oak silkworm pupa. Pyruvic acid was found in the pupa of the oak silkworm by Burova [6]. We have found fructose diphosphate in this pupa [7], pointing to the existence of the glycolytic pathway of carbohydrate metabolism in this material, and we also found active aldolase [8]. It is thus reasonable to assume that pyruvic acid is formed by the degradation of glycogen, and that it undergoes amination, to yield alanine, or is incorporated into oxaloacetic acid, which gives aspartic acid. The necessary ammonia may be derived from glutamine, which has been found by Kaplan [9] to be present in the oak silkworm. Transamination processes are also possible, since the Japanese workers Koida, Nagayama and Simura [10] have reported the presence of aminotransferase in silkworms.

The view that glycogen is degraded only as far as pyruvic acid, which is not further oxidized to carbon dioxide and water, is supported indirectly by the findings of Zolotarev, Lavrova and Tokareva [11] that the respiratory quotient of the silkworm pupa is low, being of the order of 0.45-0.6, pointing to the breakdown and oxidation of fats, rather than of carbohydrate. Zubova's studies of the metabolism of fats [12] in the oak silkworm have established that fats are intensively used up during the metamorphosis of both male and female silkworms.

It is usual to express the contents of various components as percentages of dry weight of the given material. This procedure may, however, give rise to erroneous conclusions when the dynamics of a process is under consideration. If the content of some substances falls during the course of a process, this will automatically raise the contents of other components, although these need not necessarily be involved in the given process.

In the process of transformation of a pupa into a moth we find considerable changes in the contents of various substances, such as fats and carbohydrates, resulting in the relative increase in the protein content. It is for this reason necessary, in the study of such processes, also to take into account the changes in the absolute amounts of various substances present in a single individual. We have done this for male pupae (Table 2).

A consideration of the data of Table 2 shows that the total nitrogen content varies from 101.5 to 103.9 mg, i.e. it remains at a uniform level, which is as would be expected, since the pupa does not take in or excrete any nitrogenous compounds. Protein nitrogen shows no changes until the 9th day, after which it begins to rise, achieving a value of 93 mg in one pupa by the 20th day. The increase in protein nitrogen thus amounts to 14 mg. Parallel with this is the fall in amino group nitrogen, but this amounts to only 7 mg by the 20th day; this is clear evidence that some substances other than amino acids contribute to the synthesis of proteins. Practically all of the glycogen (48.5 out of 50 mg) has been used up by the 20th day. The abrupt fall in glycogen content also begins on the 9th day, on which day definite evidence of protein synthesis is first seen.*

* When our experimental work had already been concluded two papers, by Vyskrebentseva [13] and by Sisakian and Kuvaeva [14], appeared, on metabolic processes taking place in the body cavity fluid of the mulberry silkworm. Our views as to the role of pyruvic acid in protein synthesis are in accordance with those expressed by these authors.

TABLE 2

Amounts of Total, Protein and Amino Group Nitrogen, and of Glycogen Present in a Single Individual (mole) at Various Stages of Development

Day of development	Wt of one pupa, in g	Total nitrogen, mg	Protein nitrogen mg	Amino group nitrogen, mg	Glycogen, mg
1	5,31	103,2	79,7	10,5	50,0
4	5,26	102,7	74,8	9,6	49,7
8	5,19	103,2	81,6	6,5	41,5
9	5,17	101,5	78,6	7,3	48,5
13	5,10	103,7	80,2	7,3	35,0
17	4,94	101,5	86,1	4,8	15,5
20	4,92	103,9	93,0	3,5	1,5
Moths	1,34	53,1	47,4	1,2	0,5

SUMMARY

The total and protein nitrogen contents of male oak silkworm pupae are higher than those of females on the first day of development. The protein content begins to rise from the 9-13th day of development up to the time of emergence of the imago.

Amino group nitrogen falls over the whole period of development, but this is insufficient to account for the whole of the rise in protein nitrogen, suggesting that amino acids are formed from nonnitrogenous precursors during metamorphosis.

The glycogen content falls steadily, beginning with the 9-13th day of development. It is suggested that degradation of glycogen not only serves for providing the energy requirements of protein synthesis, but also contributes intermediate substrates for this purpose.

LITERATURE CITED

[1] N. F. Pravdina and A. N. Smolin, Uchen. Zapiski MGPI im. V. I. Lenina 140, 240 (1958).

[2] N. M. Sisakian and E. B. Kuvaeva, Biokhimiia 18, 354 (1953).

[3] P. Ostern and St. Hubl, Chem. Abstr. 36, 5844 (1942).

[4] G. Gorbach, G. Dedic, and E. H. Philippi, Z. physiol. Chem. 301, 185 (1955).

[5] R. D. Gal'tsova and S. Ia. Demianovskii, Uchen. Zapiski MGPI im. V. I. Lenina 77, 25 (1953).

[6] A. A. Burova, Uchen. Zapiski MGPI im. V. I. Lenina 77, 33 (1953).

[7] A. N. Smolin, Biokhimiia 17, 61 (1952).

[8] A. N. Smolin, Uchen. Zapiski MGPI im. V. I. Lenina 98, 91 (1957).

[9] V. A. Kaplan, Ukrain. Biokhim. Zhur. 20, 193 (1948).

[10] F. Koida, H. Nagayama, and K. Simura, J. Agric. Chem. Soc. Japan 29, 987 (1955).

[11] E. Kh. Zolotarev, N. P. Lavrova, and L. V. Tokareva, Zoolog. Zhur. 19, 46 (1940).

[12] V. A. Zubova, Uchen. Zapiski MGPI im. V. I. Lenina 98, 3 (1957).

[13] E. I. Vyskrebentseva, Biokhimiia 22, 657 (1957).

[14] N. M. Sisakian and E. B. Kuvaeva, Biokhimiia 22, 686 (1957).

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CHANGES IN OXIDATION-REDUCTION POTENTIAL OF LEAF HOMOGENATES ("GREEN SUSPENSIONS") DURING ILLUMINATION

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Homogenates prepared by grinding up green leaves with water or buffer solution, followed by removal of the coarser particles by filtration through muslin or by centrifugation at low speeds, differ from whole leaves firstly in not having any structure, which appears to be one of the most important factors controlling the course of complex biochemical processes in living organisms, to ensure the necessary sequence of reactions. Because of this, such processes as photosynthesis, which involves a whole series of successive, and possibly also of parallel, stages, cannot take place in homogenates. However, the state of chlorophyll in such systems can hardly differ significantly from that in living leaves; there can now be no doubt that chloroplast suspensions, and, a fortiori, leaf homogenates, to which we give the name of green suspensions, retain the capability of carrying out some of the reactions of the photosynthetic process, in particular, the photochemical stage, which is directly connected with the presence of chlorophyll (Hill reaction) [1-3].

The direct relation of the Hill reaction to photosynthesis has been confirmed by determination of the isotope composition of the oxygen evolved, which has been found to be identical with that of the water entering into the reaction [4], by the similarity of the kinetic coefficients of both processes [5], by the identical order of the quantum yield of the Hill reaction and of normal photosynthesis [6], and to some extent by the results of experiments involving the use of poisons and inhibitors [3]. There is every reason to believe that the difference between the Hill reaction and normal photosynthesis is basically that whereas in the latter case the active hydrogen (electron) formed in the photochemical stage is accepted by an intermediate product derived from CO_2, in the former case it is accepted by an added oxidant.

This conclusion is not in principle invalidated by the finding that chloroplast preparations and leaf homogenates are able, under reducing conditions, to fix small amounts of carbon dioxide in an atmosphere of hydrogen [8], in the presence of added TPN [9], and of DPN and of a series of enzymes of the glycolytic sequence [10].

It was natural to suppose that investigation of the redox potential of illuminated green suspensions might contribute to an understanding of the process of formation of the reducing agent responsible for reduction of CO_2 in photosynthesis, or of the substitute oxidant used in the Hill reaction, and which persists in the systems after illumination in the absence of an acceptor [11]. Such green suspensions, or, as they are sometimes called, "green solutions of the colloid components of chloroplasts," have been extensively used in our laboratory, both for manometric studies [12, 13], and for spectrophotometric studies in which dichlorophenolindophenol was used as the oxidant [3].

The object of the present investigation was to find out how light absorbed by chlorophyll affects the oxidation-reduction properties of green suspensions. Does illumination change the redox potentials of such suspensions, in which direction are they shifted, and what are the conditions favoring or inhibiting this process?

For our investigations we used a potentiometric method, as previously applied by us to the study of the photochemical properties of dissolved chlorophyll [14]. A particular advantage of this method for work with such material as green suspensions was that it enabled us to measure the oxidation-reduction properties of the systems without the need for adding any other reagents to them.

PUBLISHED DATA

Although very numerous researches have been devoted to the determination of redox potentials of plant and animal tissues, very little work has been done on the changes in redox potential of biological systems containing chlorophyll following their illumination.

Such work as has been done falls into two categories: 1) work on suspensions of intact living organisms, in particular the unicellular alga Chlorella [15, 16] and the purple bacterium Chromatium D. [17], and 2) work on suspensions of chloroplasts, whole or fragmented, and washed or unwashed [18-21].

The researches of the first category are of much less interest to us, inasmuch as the observed changes in the oxidation-reduction state of the suspensions of these organisms following illumination are due not so much to direct modifications of the light-sensitive photosynthetic apparatus as to the products excreted into the medium, and resulting from the vital activities of the illuminated organisms. We shall not, for this reason, consider them in detail. We would only remark that the authors of these papers come to the conclusion, based on more or less direct data, that illumination of the plant organism leads to formation of electrode-active substances which appear to possess a higher reduction potential than that found before illumination.

Spikes et al. [20], whose paper belongs to the second category, used suspensions of spinach chloroplasts to which sucrose and an oxidant [$K_3Fe(CN)_6$] had been added, and these were illuminated under aerobic conditions. This research can be considered as amounting to a study of the course of the Hill reaction, based on the changes in the redox potential of the suspensions, which were in the first place determined by the amount of oxidant added. The only experiment performed by these authors without added oxidant, using a solution of sunflower leaf cytoplasm, showed that the redox potential of the medium shifted in the direction of reduction during illumination, subsequently reverting to the initial potential in the dark. As has been shown by the results of our experiments, such an effect is possible under aerobic conditions only under conditions of strong illumination, with restricted diffusion of oxygen into the solution, and such conditions did in fact apply in these experiments.

Gerretsen [18] found that illumination of oat chloroplast suspensions led to a rise in redox potential under aerobic conditions. He ascribed this effect to the hydrogenation of oxygen to give hydrogen peroxide, which is formed during illumination by reduced products. The reaction appeared to be activated by manganese ions. Of particular interest to us are those very few experiments of this author which were conducted under anaerobic conditions, or with restricted access of oxygen. In these, he found that the potential became more negative.

Gilmour, Lumry, and Spikes [21] measured the changes in potential of a platinum electrode placed in suspensions of washed, fragmented sugar beet or spinach chloroplasts, under substantially anaerobic conditions. These authors regard the electrode activity of the reduced products formed as being analogous to the transfer of hydrogen (electrons) in the Hill reaction. By surrounding the electrode with a semipermeable colloidal membrane, these authors were able to show that the observed changes in electrode potential were due not to small water-soluble molecules, but to substances which were firmly attached to the chloroplast fragments, and were on the surface of insoluble particles. This is of particular interest in view of the findings of Thomas et al. [22], that the size of the chloroplast fragments can be very considerably reduced without abolition of photochemical evolution of oxygen by the Hill reaction. The photochemical reaction, and those associated with it, do not appear to require the presence of macrostructure, and they proceed with microstructural formations so small as to contain not more than a few hundred molecules of chlorophyll-protein complex. It is quite probable that such reactions take place at the interfaces of protein – chlorophyll lamina, the existence of which has been demonstrated by numerous electron microscope studies. Gilmour and his co-workers believe that photoreduction involves the participation of two substances, which are able to react with each other, but only one of which shows activity at the electrode.

Spruit and Wassink [19] have attempted an interesting parallel study of oxidation-reduction potential changes and of variations in the fluorescence of chlorophyll in Chlorella suspensions.

Their findings permit of the conclusion that reducing substances are formed in the immediate vicinity of chlorophyll, during illumination of the suspensions. During the induction period the shape of the potential change and fluorescence change curves is not affected by presence of CO_2, suggesting that the observed phenomena are related to the primary photochemical reactions, and not to the secondary dark reactions of photosynthesis.

It may thus be assumed, on the strength of the available data, that a direct effect of light is to shift the oxidation-reduction potential of chloroplast suspensions or leaf homogenates to more negative, i.e. reducing, values. The available experimental material is, however, too scanty, and is derived under a variety of conditions; the problem requires more thorough and more precisely planned investigation.

EXPERIMENTAL MATERIAL AND APPARATUS

For our experiments we used suspensions prepared by grinding up green leaves with water or phosphate buffer solution, and straining the suspension through a few thicknesses of muslin or canvas (in some cases we applied centrifugation at 2000-3000 g). We usually took 1.2-1.5 g of leaves for each experiment, first washing them free of surface contamination (the particles were removed). The suspensions were prepared at room temperature, in dim light. We did not think it necessary to carry out the preparation of the suspensions at low temperature, since we were concerned primarily with the action of light on the chlorophyll complex in its natural state, so that it was even desirable, before taking the suspensions for the experiments, to have achieved equilibrium, in the shortest time possible after conclusion of the enzymatic reactions of an oxidative-reductive nature which inevitably result from the grinding up of living material.

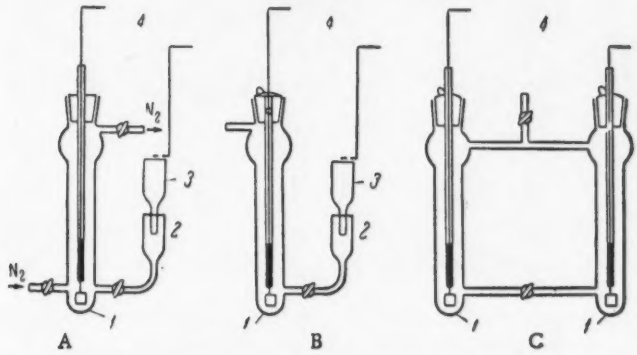

Fig. 1. Electrode vessels for measurement of oxidation-reduction potential changes during illumination.
1) Platinum electrode; 2) tube containing saturated KCl solution; 3) calomel electrode; 4) leads to the potentiometer.

Figure 1 shows three of the electrode vessels used by us. The vessels are so designed as to make it possible to remove oxygen from the fluids by passing nitrogen, freed of oxygen by passage through a number of wash-bottles containing alkaline sodium hydrosulfite solution (vessel A), or by degassing with a vacuum pump for 5-6 min , with energetic shaking (vessels B and C). The tap of vessel B was made gas-tight by sealing with a special grease prepared from agar and potassium chloride soltuions [14, 23].

As reference electrodes we took either calomel electrodes (vessels A and B) or a platinum electrode placed in an unilluminated solution (vessel C). The light source was either a 300 W lamp with a glass condenser, or a 75 W bulb with a matt reflector, at a distance of 6 cm from the vessel. Infrared radiation was filtered out by means of a glass filter. Except where the experiments required it, a red filter (RG-1 or RG-2) was interposed between the light source and the vessel, so as to cut off light of frequencies other than those absorbed exclusively by chlorophyll. The potential differences between the reference electrode and the indicator electrode placed in the illuminated suspensions were measured with a MOSKIP (LP-5) potentiometer, to an accuracy of 1-2 mV.

EXPERIMENTAL RESULTS

In our preliminary experiments we investigated the effect of illumination on the potential of a smooth platinum electrode dipping into suspensions prepared in the above-described way from leaves of various plants (sugar beet, bean, acacia, cucumber, lilac, nettle, oat, wheat, dandelion, etc.). The experiments were performed

with buffered (pH 6-8) and unbuffered suspensions, from which the coarser particles had been removed in various ways, and using different electrodes and electrode vessels. Without entering into a detailed discussion of the results obtained, we would only record that in all the experiments involving illumination of the suspensions under anaerobic conditions the potential became more negative. The magnitude of this shift varied very considerably, however, for different plants. The largest shifts were found with suspensions of sugar beet and bean leaves, and for this reason nearly all of our basic experiments were performed on this material. We present the results of three experiments conducted at room temperature (Fig. 2), as illustrations of the typical changes encountered in the potentials of suspensions during illumination.

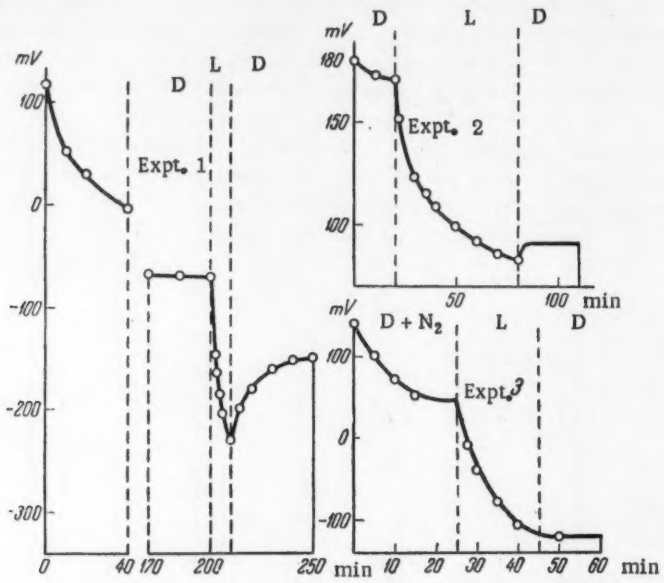

Fig. 2. Effect of illumination on the oxidation-reduction potential of green suspensions.
Experiment 1) Bean leaves in water, vessel 2, without removal of air. Experiment 2) Bean leaves in phosphate buffer at pH 7, vessel 2, air pumped out. Experiment 3) Sugar beet leaves in water, vessel 1, swept out with N_2. Ordinates – potential in mV, with reference to a calomel electrode. In this and in subsequent graphs: D) dark and L) light.

We did not remove air from the vessel in Experiment No. 1, but access of atmospheric oxygen to the tube was restricted by its narrow lumen. As is evident from the figure, the oxidation-reduction potential of the suspension fell before exposure to light, at first rapidly, and then more slowly, until a steady state was reached, in which the curve ran nearly parallel to the time axis. This fall in potential, proceeding in the dark, may be ascribed to enzymatic and nonenzymatic chemical processes proceeding in freshly ground leaves; of these reactions, those involving the action of oxidases are of special importance, as they lead to the oxidation of readily oxidizable substances, and hence to the rapid utilization of dissolved oxygen. Owing to the restricted diffusion of air into the bulk of the suspension we may suppose that, after the lapse of a certain period of time, anaerobic conditions will prevail in the vicinity of the electrode. The potential of the platinum electrode fell sharply after switching on the light, and after switching it off it gradually became more positive. This rise in the potential curve may be ascribed to gradual diffusion of oxygen into the suspension, in amounts sufficient to oxidize the reducing substances formed during illumination.

The results of experiments Nos. 2 and 3 (Fig. 2) are very similar to the above. The smaller initial fall in potential before illumination, and the smaller rise after cutting off the light, or even the absence of such a rise, are due to the elimination of oxygen before the commencement of the experiment.

We thought it desirable to demonstrate the participation of chlorophyll in this process by means of even more direct experiments, although the above-described changes in potential of the suspensions were observed using red light, which is in itself evidence of the direct part played by chlorophyll, since this is the only pigment present in the given plant material which absorbs light in the red part of the spectrum. With this object, we performed experiments with etiolated leaf suspensions. It is evident from Fig. 3 that curves 1 and 2 are unaffected by presence or absence of light, whereas curve 3, representing the results given by suspensions containing an appreciable amount of chlorophyll, shows clear evidence of the effect of light on the potential.

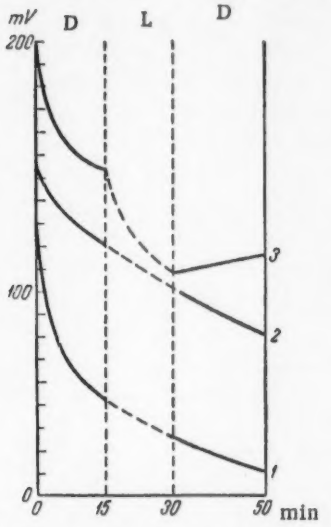

Fig. 3. Effect on the oxidation-reduction potentials of illumination of suspensions of etiolated bean leaves (in water, as mV with reference to a calomel electrode). 1) Grown in complete darkness; 2) exposed to light (1000 lux) for 3 hours; 3) the same, for 12 hours.

It should be noted that the validity of these experiments is not affected by differences in the initial potentials of the suspensions. These are due, in the first place, to the impossibility of ensuring completely reproducible surface states of the electrode. The importance of the role of chlorophyll in changing the potentials was also confirmed by experiments in which the red filter was replaced by a green one, which transmitted light of frequencies which are not appreciably absorbed by chlorophyll. Under these conditions, the effect of illumination was much smaller than with illumination with red light of the same intensity.

As we have pointed out, the experimental results cited above are only examples of those obtained by us in a large number of experiments. A characteristic feature of active green suspensions is that, when illuminated under anaerobic conditions with light of a wavelength absorbed by chlorophyll, their oxidation-reduction potential falls (becomes more negative), but never rises. The shifts toward more positive potentials of suspensions of whole cells or chloroplasts, reported by some of the above-mentioned authors, may be ascribed to secondary effects, in particular those due to the action of oxygen, which will be discussed below.

What is the nature of the compounds forming during illumination of the suspensions, and affecting the potential of an inert electrode, and how are they related to the photosensitive chlorophyll – protein (lipid) complex? Are they directly combined with this, or are they transients entering into the chain of photosynthetic reactions at some point remote from it? We performed a special series of experiments, with the object of answering these questions.

The results of experiments in which the suspensions were exposed to light very briefly supported the view that the electrode-active reducing compounds appearing are either primary products of the photochemical reaction of chlorophyll, or are not far removed from them; the potential fell as soon as the light was switched on, without any induction period, pointing to the immediate appearance in the suspensions of electrode-active products.

These products readily react with oxygen. This was shown by the reversibility of the potential changes in experiments with restricted access of oxygen. Saturation of the suspensions with oxygen during exposure to light, by bubbling air through them, gave various results, depending on the state of the suspensions and the intensity of illumination. With weak illumination of active suspensions there was a noticeable potential rise. If the intensity of illumination was sufficiently high we found that the potential either remained at a steady level, or else fell, although to a lesser extent than under anaerobic conditions. In this case, production of reducing substances proceeds so rapidly that the equilibrium point of the two opposed processes is shifted in the direction of reduction.

This observation is evidence that, even under aerobic conditions, minute regions may exist, in the presence of microstructures, in which anaerobic conditions prevail, and in which reducing reactions are possible. Such conditions are even more likely to be present in a living leaf, with its intact structure, and this is the probable explanation of the fact that both anerobic and aerobic processes may proceed simultaneously in leaves.

The maximum potential rises observed when oxygen is bubbled through the suspensions during exposure to light are appreciably greater than when oxygen is passed without illumination. It is evident that the reactions

of the products with oxygen lead to production of peroxidic compounds possessing a higher oxidative potential than does dissolved molecular oxygen [18]. This is illustrated by the curves of Fig. 4, which show the effect of previous illumination on the level of the potential reached when air is subsequently passed through the suspension.

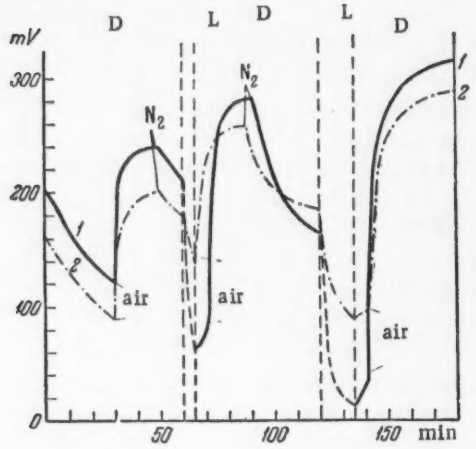

Fig. 4. Effect of illumination on the potential level reached when air was subsequently passed through the suspension (see text).
1) Sugar-beet leaf suspension in water; 2) the same, with oat leaves.

Nitrogen was passed through the suspension for 30 min , in the dark, followed by air, for 20 min , also in the dark. Nitrogen was then again passed, and the light was switched on at the same time, for 5 min. Air was then passed. Nitrogen was then again passed for 30 min , followed again by air and illumination, but the duration of the exposure to light was now 15 min. The potential differences between the indicator and the reference electrodes were read at frequent intervals throughout the experiment.

We examined the effects of a number of factors on the potential changes taking place when green suspensions were illuminated; these factors were temperature, ultraviolet irradiation, and enzyme poisons and narcotics.

Suspensions prepared from leaves which had been immersed in water at 80° for 5 min gave no potential changes on illumination. The effect of temperature on already prepared suspensions was even greater. As is shown by Fig. 5, A, heating the suspensions at 35° and 40° did not abolish their capability of changing the oxidation-reduction potential during exposure to light, whereas heating at 50° resulted in their total inactivation, although there was no visible evidence of coagulation of the suspensions.

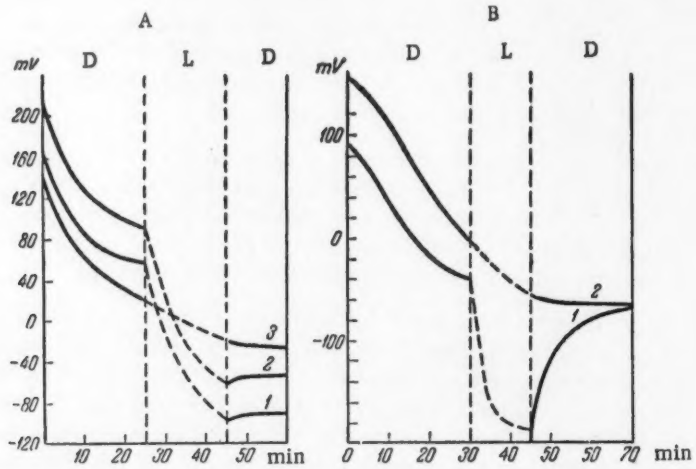

Fig. 5. Effects of various factors on the photoelectrochemical activity of green suspensions prepared from sugar-beet leaves.
A) Suspension heated for 30 min : 1) at 35°; 2) at 40°; 3) at 50°.
B) Irradiation with a quartz – mercury vapor lamp: 1) ordinary suspension; 2) suspension irradiated for 1 hour.

Although cooling at 0° did not irreversibly inactivate the suspensions, photoelectrochemical activity was not seen during illumination at this temperature. This is illustrated by the results of the experiment shown in

Fig. 6. The slope of the potential curve is unaffected by illumination at 0°, whereas at 25° it dips sharply when the light is turned on. It is of interest that cooling alone causes a lowering of potential, whereas rapid rewarming to room temperature has the opposite effect. In view of this, it cannot be argued that the potential drop during illumination is in no way connected with the oxidation-reduction state of the suspensions, but is due to localized temperature rises due to absorption of the energy of the incident light. Were this to be the case, the effect observed would be the reverse of what is actually seen, i.e. we should expect a rise in potential, rather than a fall.

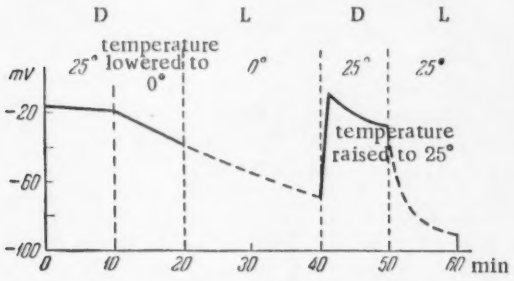

Fig. 6. Photoelectrochemical activity of green suspensions prepared from sugar-beet leaves (in water), at 0° and 25°.

A number of authors [24-26] have shown that ultraviolet irradiation of chloroplast suspensions lowers their photochemical activity, as shown by their ability to reduce certain substances during illumination. This inactivating effect is not due to destruction of chlorophyll, but is caused by inactivation of various enzymes, which are, according to Sisakian's findings [27], very numerous in the chloroplasts. As appears from Fig. 5, B, exposure of an ice-cooled sugar-beet leaf green suspension to an unscreened PRK-2 quartz – mercury vapor lamp at a distance of 35 cm for 1 hour led to the abolition of its photoelectrochemical activity.

Thus, the results of experiments on the effects of temperature and ultraviolet irradiation on the formation in green suspensions of products which lower the oxidation-reduction potential of the suspensions indicate that enzyme systems take part in the process of formation of these substances. The observed lowering of potential is evidently not due directly to the purely photochemical reaction of the photosensitive chlorophyll complex, but depends also on enzymatic reactions which proceed in the absence of light. In order to get some idea of which of the component reactions of the photosynthetic process is concerned in this, we examined the effect on the photoelectrochemical activity of green suspensions of some catalyst poisons and narcotics, viz., hydroxylamine, monoiodoacetic acid, sodium azide, potassium cyanide, and phenylurethane. The experiments were performed on sugar-beet leaf suspensions in vessel C (Fig. 1), pumping out the air with a vacuum pump. The inhibitors were added to the suspensions as solids or as solutions. Exposure to light was short, not exceeding 2.5 min. The potentials were measured every 15 sec. For each concentration of inhibitor taken we set up a control system with the same suspension, but without inhibitor. Our experiments gave the following results. Hydroxylamine, which is believed to block the enzyme responsible for evolution of oxygen, abolishes the ability of the suspension to lower the oxidation-reduction potential during illumination, almost completely when present in a concentration of 10^{-4} M, and totally at 10^{-3} M.

Total inhibition of photoelectrochemical activity of green suspensions was effected by 10^{-2} M monoiodoacetic acid. This inhibitor is believed by a number of authors [1] to inactivate catalase, and to react with SH-groups of proteins.

Azide, which is a powerful inhibitor of photosynthesis, and which has considerably less effect on the reaction with chloroplasts [1], depressed the photoelectrochemical activity of green suspensions at concentrations of not less than 10^{-2} M, but did not abolish it even at this concentration.

The action of cyanide differs according to the plant material taken. In some cases it powerfully inhibits photosynthesis, whereas in others the same concentrations not only do not inhibit this process, but they even stimulate it. The majority of workers [1] are of the opinion that cyanide does not interfere with the actual photosynthetic reactions, but that it acts on the enzyme or enzymes concerned in the dark reaction of photosynthesis. Cyanide does not suppress the reactions effected by isolated chloroplasts, which are concerned chiefly with the photochemical stage of photosynthesis. Our experiments on sugar-beet leaf suspensions gave the following results: potassium cyanide at a concentration of 10^{-4} M caused a perceptible stimulation of the photoelectrochemical effect. Some inhibitory effect was found with 10^{-3} M cyanide, and very considerable inhibition with 10^{-2} M cyanide. However, even at this relatively high concentration total inhibition was far from being achieved.

The photoelectrochemical activity of green suspensions was partly inhibited by 10^{-3} M, and totally inhibited by 10^{-2} M phenylurethane.

It may be concluded, from a consideration of our findings as to the effects of inhibitors on the photoelectrochemical activity of green suspensions, in conjunction with published data on inhibition of the Hill reaction, that the action of the inhibitors is practically identical in both cases. Those inhibitors which had been found to be active in the Hill reaction were equally effective in suppressing photoelectrochemical activity, and, conversely, substances not affecting the Hill reaction, such as cyanide, had very little effect on photoelectrochemical activity.

We followed the photoelectrochemical effect on sugar-beet leaf suspensions over the whole of the vegetative period, in order to investigate the effects of the age and the environmental growth conditions of the plant. We found that the absolute value and the rate of fall of the potentials of suspensions during illumination were powerfully influenced by the environmental conditions of the plant at the time when the leaves were taken from it. The response of the oxidation-reduction potential to illumination was noticeably greater with material collected toward the end of the vegetative period, or even slightly affected by frost, than with leaves from earlier growth stages, which is in accordance with the findings of Andreeva and Zubkovich [28].

SUMMARY

It may thus, on the basis of published data and of our experimental findings, be taken as definitely established that the oxidation-reduction potential of active green suspensions becomes more negative when they are exposed to light under anaerobic conditions.

The results of experiments on the effects of temperature, ultraviolet irradiation, and various inhibitors indicate that the process of formation of reducing electrode-active substances is not a purely electrochemical reaction, but also involves a dark, probably enzymatic, stage. The powerfully depressing action of such inhibitors as are known to suppress processes connected with evolution of oxygen from water, and hence with production of active hydrogen or electrons, is evidence that the electrode-active reducing substances are formed in the suspensions as a result of transfer of this hydrogen or electron through chlorophyll to a suitable acceptor, which is present in the suspension.

Our findings support the view that photochemical acceptance of an electron (or hydrogen atom) by chlorophyll in its natural state (according to the concepts developed in our laboratory, this represents the primary step of photosynthesis [14, 29]) does not take place directly from water ('photolysis of water'), but passes through a succession of 'preparatory' enzymatic reactions, in which water, as the electron donor, is bound to natural chlorophyll. It is quite possible that the direct donor of electrons to natural chlorophyll may be a compound resembling the reduced cytochromes [7, 31], having a long-lived excited state, and able to react in the primary photoreductive step.

LITERATURE CITED

[1] E. Rabinowitch, "Photosynthesis," Vol. I; Vol. II, Part 1 [translation from English] Izd. IL, Moscow, 1951, 1953).

[2] E. Rabinowitch, "Photosynthesis," Vol. II, Part 2 (Interscience, New York, 1956).

[3] L. M. Kosobutskaia, "Study of the spectral and photochemical properties of chlorophyll in its natural state" Thesis (Moscow, 1954).

[4] A. S. Holt and C. S. French, Arch. Biochem. 19, 429 (1948).

[5] H. C. Ehrmantraut and E. Rabinowitch, Arch. Biochem. Biophys. 38, 67 (1952).

[6] E. C. French and G. S. Rabideau, J. Gen. Physiol. 28, 329 (1945).

[7] R. Hill and C. P. Whittingham, Photosynthesis (London, 1955).

[8] E. A. Boichenko and V. I. Baranov, Doklady Akad. Nauk SSSR 91, 339 (1959).*

[9] J. L. Tolmach, Nature 167, 946 (1951).

*As in original – Publisher.

[10] W. Vishniac and S. Ochoa, J. Biol. Chem. 195, 75 (1952).

[11] A. A. Krasnovskii and L. M. Kosobutskaia, Doklady Akad. Nauk SSSR 82, 761 (1952).

[12] G. P. Brin and A. A. Krasnovskii, Biokhemiia 16, 453 (1951).

[13] G. P. Brin, "Participation of chlorophyll in hydrogen transfer processes" Thesis (Moscow, 1956).*

[14] V. P. Evstigneev, "Oxidation-reduction properties of chlorophyll, in relation to its role in photosynthesis" Thesis (Moscow, 1936).*

[15] P. S. Tang and C. G. Lin, J. Cell Comp. Physiol. 9, 149 (1936).

[16] C. J. P. Spruit, Acta bot. Neerl. 1, 555 (1953).

[17] E. C. Wassink et al., J. Microbiol. 12, 281 (1947).

[18] F. C. Gerretsen, Plant and Soil 2, 159 (1950); 3, 1 (1951); Nature 171, 207 (1953).

[19] C. J. P. Spruit and E. C. Wassink, Biochim. biophys. acta 15, 357 (1954).

[20] J. D. Spikes, R. Lumry, H. Eyring, and R. Wayrynen, Arch. Biochem. 28, 48 (1950).

[21] H. S. A. Gilmour, R. Lumry, and J. D. Spikes, Plant Physiol. 28, 89 (1953).

[22] J. Thomas, O. Blaaw, and S. Duysens, Biochim. biophys. acta 9, 575 (1952); 10, 230 (1953).

[23] V. B. Evstigneev and V. A. Gavrilova, Doklady Akad. Nauk 92, 381 (1953).

[24] A. S. Holt, A. C. Newton, and W. A. Arnold, Am. J. Bot. 35, 810 (1948).

[25] A. S. Holt, J. A. Brooks, and W. A. Arnold, J. Gen. Physiol. 34, 627 (1951).

[26] I. V. Vereshchinskii and A. A. Krasnovskii, Biokhimiia 16, 621 (1951).

[27] N. M. Sisakian, "Enzymatic activity of protoplasmic structures" Izd. AN SSSR, Moscow, 1951).*

[28] T. F. Andreeva and L. E. Zubkovich, Trudy inst. Fiziol. rastenii AN SSSR 8, 67 (1953).

[29] A. A. Krasnovskii, Zhur. Fiz. Khim. 30, 968 (1956).

[30] A. N. Terenin, "Photochemistry of dyestuffs" Izd. AN SSSR, Moscow, 1947). *

[31] A. A. Krasnovskii, Doklady Akad. Nauk SSSR 103, 283 (1955).

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* In Russian.

IS UREA AN IRREVERSIBLE END PRODUCT OF NITROGEN METABOLISM IN THE ANIMAL ORGANISM?

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In a previous study [1] we showed the presence in gastric mucous membrane of the process of combination of ammonia and glutamic acid to give glutamine. This was accompanied by decreased gastric secretion and formation of hydrochloric acid.

In the present paper, in continuation of the previous study, we give data on the origin of ammonia in the gastric mucous membrane, the content of which is increased at the time of stimulation.

EXPERIMENTAL

In 20-30 min after subcutaneous injection of 5 mg of histamine, the animals (cats) were exsanguinated; the stomach was removed and carefully washed, and the gastric mucosa separated; this was at once ground with a 6 % solution of trichloroacetic acid in the ratio 1 : 9 and the resulting homogenate was centrifuged. The amount of ammonia in the centrifugate was determined according to Conway. Ammonia was also determined in the gastric mucous membrane of control animals which had not received histamine.

The great variation in ammonia content in both series of experiments was explained by its rapid formation in enzymatic action after death. This explains the difficulty in direct determination of ammonia in gastric mucosa. It is impossible here to use rapid freezing of the whole stomach in liquid oxygen to depress the enzyme processes, since it then becomes impossible to separate the mucous membrane. Nevertheless, the data confirm the increased amount of ammonia in the secretory apparatus of the stomach when it is stimulated by histamine (Table 1).

There is also a question of the origin of the ammonia formed in the gastric mucous membrane. We have expressed the opinion [2, 3] that the source can be, in addition to glutamine, also urea, which agrees with the fact of the presence of urease in the stomach, shown by Luck [4]. Therefore, we determined ammonia in gastric mucous membrane after subcutaneous injection of a $CdCl_2$ solution, which, as is known, is a strong urease inhibitor. This decreased the amount of ammonia on the average by 34% compared to the control animals (Table 1).

However, recently a number of investigators (Kornberg and Davies [5], Heinz and Obrink [6], Epshtein [7], and others) have denied that urease is an intracellular enzyme of gastric mucous membrane, considering it as a bacterial enzyme of organisms which occupy the stomach and upper respiratory tract. Hence, as noted by a number of investigators also using the method of labeled atoms [8, 9, 10] the splitting of urea does not occur at all in the animal cells themselves, but is the result of the life activities of the microflora which live in them. It is true Conway does not share the opinion of Kornberg and Davies about the bacterial nature of stomach urease [11].

Thus, there are two contradictory points of view on this question which makes it important to investigate further.

Corresponding experiments were made on dogs with small Pavlov gastric pouches and fistulas of the main stomach. In the first series of experiments with dogs, a solution of urea (1 : 10) was introduced through the fistula of the main stomach, and after 20 min a food stimulant was given (100 g of meat). In hourly portions of the gastric juice collected over 4 hours, the ammonia was determined according to Parnas, the urea content was determined by the xanthydrol method, and the acidity according to Michaelis. In the control experiments, instead of a urea solution, an equal volume of distilled water was introduced. The results are given in Table 2.

TABLE 1

Change in Amount of NH_3 in Gastric Mucous Membrane of Cats with Stimulation of Gastric Secretion by Histamine and After Parenteral Administration of $CdCl_2$

Expt. No.	Control	After histamine	After $CdCl_2$		
	NH_3, mg%	NH_3, mg%	amount of $CdCl_2$ in ml		NH_3, mg%
1	17,6	15,5	9	0,5*M*	18,9
2	7,4	14,5	15		16,6
3	28,9	37,7	10		8,7
4	27,4	28,5	8		22,4
5	27,1	24,4	10	2*M*	3,4
6	21,0	29,7	10		12,7
7	—	22,9	10		16,7
8	—	46,1	10		18,1
9	—	—	10		9,8
Average	21,6	27,4			14,2
% Change		+26,8			—34,2

TABLE 2

Changes of Urea in the Stomach in Connection With Changes in Chemical Composition of Gastric Juice and Its Secretion

Name of animal and nature of expt.	Date	Hrs of secretion	Latent period in min	Amount of juice in ml	NH_3 mg/hr	Urea mg/hr	Free HCl in ml 0,1 N solution per 100 ml of juice
"Pontu" Control	22.II 1957	1	5	5,9	0,175	0,05	96
		2		5,2	0,190	0,15	108
		3		3,4	0,160	0,20	92
		4		2,4	0,142	0,15	84
Urea (5 g)	25.II 1957	1	3	8,8	0,473	0,41	108
		2		6,6	0,484	0,39	112
		3		6,0	0,440	0,47	112
		4		4,6	0,338	0,32	104
"Tommy" Control	23.II 1957	1	7	9,3	0,146	0,14	88
		2		4,2	0,101	0,12	104
		3		2,5	0,103	0,10	92
		4		2,2	0,099	0,10	80
l Urea (5 g)	26.II 1957	1	5	16,3	1,053	0,29	116
		2		5,8	0,534	0,21	120
		3		6,3	0,687	0,37	108
		4		4,5	0,504	0,24	100

As the results in Table 2 show, after introduction of urea, the amount of ammonia in the gastric juice increased, which corresponded to a strengthened stimulation of secretory function of the stomach: increase in

amount of gastric juice and its acidity, shortening of the latent period. The amount of urea in the gastric juice also increased.

In Table 3 we give the results of experiments which showed that when twice the amount of urea was introduced, the amount of ammonia in the gastric juice almost doubled, corresponding to strengthened secretion of gastric juice and its acidity.

TABLE 3

Effect of Different Amounts of Urea on the Amount of Ammonia in Gastric Juice and Its Acidity (Dog "Tommy")

Hours	5 g urea			10 g urea		
	amt. of juice in ml	NH_3 mg/hr	free HCl in ml 0.1 N solution per 100 ml juice	amt. of juice in ml	NH_3 mg/hr	free HCl in ml 0.1 N solution per 100 ml juice
1	9,9	0,78	124	13,4	1,84	140
2	6,8	0,64	132	10,1	1,37	144
3	7,7	0,80	132	9,8	1,37	132
4	6,7	0,76	124	7,8	1,22	123

These results indicate that the increased amount of ammonia in the gastric juice depends on enzymatic splitting and is closely connected with gastric secretion.

TABLE 4

Binding of Ammonia by Glutamic Acid and Resultant Inhibition of Gastric Secretion

Name of animal and nature of experiment	Date	Hrs of secretion	Latent period in min	Amt. of juice in ml	NH_3 mg/hr	Free 0.1 N HCl in ml per 100 ml juice
"Tommy" Control	2.V 1957	1	7	8,2	0,148	96
		2		3,9	0,119	104
		3		2,5	0,090	88
		4		2,9	0,080	88
Urea (5 g)	4.V 1957	1	3	15,2	0,986	116
		2		8,1	0,593	128
		3		9,7	0,651	128
		4		4,7	0,391	120
Glutamic acid (3 g), urea (5 g)	8.V 1957	1	14	4,0	0,129	—
		2		3,8	0,228	100
		3		2,7	0,220	112
		4		3,5	0,301	108
"Tippa" Control	17.V 1957	1	14	4,7	0,096	92
		2		2,5	0,075	108
		3		1,9	0,063	88
		4		1,0	0,034	88
Urea (3 g)	20.V 1957	1	10	10,3	0,217	100
		2		5,9	0,163	136
		3		6,1	0,215	134
		4		5,2	0,175	120
Glutamic acid (5 g), urea (3 g)	31.V 1957	1	18	1,6	0,049	72
		2		3,5	0,143	120
		3		3,7	0,186	124
		4		2,2	0,114	112

To settle the question of the place where the urea is split, we ran experiments on binding the ammonia with glutamic acid in the stomach.

Sodium L-glutamate was injected subcutaneously into dogs in amounts of 3-5 g calculated as the pure acid in a 1% solution of novocaine (3 g in 20 ml).

Special control experiments showed that a single injection of such an amount of novocain did not affect gastric secretion by lowering it. Fifteen minutes after the flutamic acid had been injected, a solution of urea was introduced through the main stomach fistula and after 20 minutes the dog received 100 g of meat.

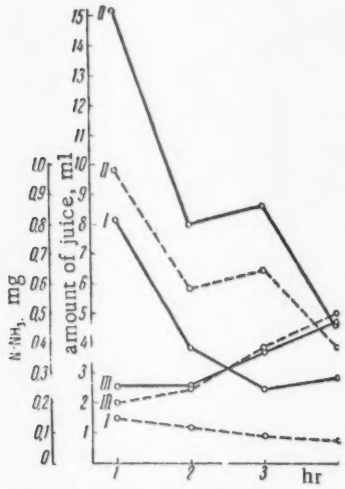

Fig. 1. Binding by glutamic acid of the ammonia formed from urea (dotted lines) and suppression of gastric secretion (solid lines): I) control, II) with urea, III) with glutamic acid and urea. Dog "Tommy".

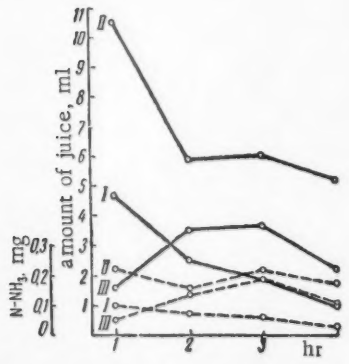

Fig. 2. Binding by glutamic acid of ammonia formed from urea (dotted lines) and suppression of gastric secretion (solid lines): I) control, II) with urea, III) with glutamic acid and urea. Dog "Tippa."

The results are given in Table 4. As in the previous experiments, the introduction of urea alone without glutamic acid caused a strong increase in the amount of ammonia in the gastric juice and a corresponding increase in its secretion and acidity and a shortening of the latent period compared to the control experiments with meat alone. Previous injection of glutamic acid strongly lowered the amount of ammonia in gastric juice and was accompanied by decreased secretion and lowered acidity of the gastric juice. The latent period also increased, that is, neuroreflex stimulation of secretory function was suppressed. These effects are especially clearly demonstrated in graphic form (Figs. 1 and 2).

DISCUSSION OF RESULTS

The results of these experiments confirm our assumption that the source of the ammonia produced by stimulation of gastric secretion is the enzymatic splitting of urea, but analysis is required to solve the problem of the location of this process.

Along with our idea of the formation of ammonia in the cells of the gastric mucosa by deamination of urea with intracellular urease there is also the theory that urea is split only after its separation from the gastric mucous membrane, and under the influence of bacterial urease. In this case it would be necessary to assume that the ammonia which was formed showed its stimulating action on gastric secretion by absorption from the stomach into the cells of the mucous membrane. However, it seems to us that intracellular splitting of urea by the urease of the gastric mucosa cells themselves is indicated by the decreased amount of ammonia in gastric juice when glutamic acid is injected.

In summary, we can assume that in the mucosa cells formation of ammonia from urea does not occur, but glutamic acid binds this ammonia which enters them from the stomach. However, it is not clear how this could cause a decreased amount of ammonia in gastric juice when this is formed over a long period from urea under the action of bacterial urease.

This could occur only if there was a corresponding decrease in intensity of this process.

However, it is difficult to assume such a correlation of binding ammonia within the cell by glutamic acid and intensity of its formation in the stomach. The results of Conway [11] also indicate the presence of urease in the cells of the gastric mucous membrane since they show that protoplasm removed by a microcannula from cells of the gastric mucosa has urease activity, while in the intercellular substance this is not found.

Thus, our experiments with glutamic acid and the data of Conway strengthen the assumption of the presence of an intracellular urease in gastric mucous membrane. This evidently does not exclude the possibility

of bacterial splitting of urea in the stomach, and we are at present studying this. In whichever of these two ways ammonia is formed from urea, it is bound by glutamic acid in the cells of the gastric mucous membrane and is thus again drawn into the circle of biochemical processes; therefore, urea cannot be regarded as an irreversible end product of nitrogen metabolism.

LITERATURE CITED

[1] E. I. Martinson and Kh. Lind, Biokhimiia 20, 533 (1955).

[2] E. E. Martinson, Trudy Nauch. Sessii AN ESSR, Tallin (1949).

[3] E. Martinson, Biokhimiia 15, 121 (1950).

[4] J. M. Luck, Biochem. J. 18, 814 (1924).

[5] H. L. Kornberg and R. E. Davies, Physiol. Rev. 35, 169 (1955).

[6] E. Heinz and K. Obrink, J. Physiol. Rev. 34, 4, 643 (1954).

[7] Ia. A. Epshtein, Usp. Sovr. Biologii 43, 29 (1957).

[8] E. Leifer, L. J. Roth, and L. H. Hempelman, Science 108, 748 (1948).

[9] H. L. Kornberg and R. E. Davies, Biochem. J. 52, 345 (1952).

[10] Chao Fu-Chuan and H. Tarver, Proc. Soc. Exp. Biol. Med. 84, 406 (1953).

[11] E. J. Conway, Twentieth Congres international de physiologie, (Bruxelles, 1956), p. 159.

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THE ACTION OF IONIZING RADIATION ON SOME NONPROTEIN THIO-COMPOUNDS OF THE ANIMAL ORGANISM

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In recent years many investigations have convincingly shown that the most vulnerable components of cells to ionizing radiation are the nucleic acids and the SH groups. As a result of its action on nucleic acids there is a decrease in the content of both nucleic acid fractions: desoxyribonucleic acid (DNA) and ribonucleic acid (RNA), and first DNA [1, 2, 3]. The action on SH groups produces more or less inactivation of the thiol enzymes in experiments in vitro [4, 5]. While we agree fully with the first point of view, we consider it doubtful that the irradiation factor directly or indirectly acts on the SH groups of the organism. In a previous study [6] we investigated the effect of total irradiation of rats by X-rays on some thioenzymes. It was shown that the catalytic activity of tissue dehydrogenase and cathepsin and also their sensitivity to thiol poisons was higher in irradiated rats than in normals. On this basis we concluded that the reactivity of SH groups was increased in irradiated animals. Previously, we studied only the SH groups of proteins. The problem of the present work is to study the effect of x-rays on the thio groups of nonprotein substances. Since the chief nonprotein thiol compounds of the organism are glutathione and coenzyme A (CoA), these were the objects of our investigation.

There is only a very limited amount of work in the literature which relates directly to the question which interests us. Thus, Patt, Straube et al. [7] concluded that rats irradiated with x-rays in doses of 200-800 r showed no particular change in the amount of SH groups of blood serum. Analogous results were obtained by Graevskii [8] in a study of the amount of SH groups in tissues of liver, kidney, and spleen in mice irradiated with 700 r. In studying the effect of irradiation by x-rays, Van-Hemyngen [9] showed that in the lens of an irradiated rabbit eye the CoA concentration fell sharply compared to the normal. On the other hand, in a study of the change in acetylation processes in irradiated animals, some authors have not observed any change in intensity of these and so have concluded that CoA activity is not changed by the action of ionizing radiation [10-12]. The work of Bacq and Herve [13] deserves mention, in which was shown a certain protective action on CoA under the influence of ionizing radiation, and the complete absence of medical effects.

METHODS

As experimental animals we used white rats weighing 160-180 g which were irradiated with an absolutely lethal dose of x-rays (1200 r) and sacrificed after 24 and 48 hours. In the control and experimental animals we measured changes in CoA activity and content of reduced glutathione. At the same time we studied changes in sensitivity of CoA to arsenic-containing thiol poisons. The glutathione content was determined by the method of Wachguider and Quensel [14]. As is known, this method is not specific for reduced glutathione, since several substances and other reducing groups present in tissues are here determined. We considered it possible to use this method since, on the one hand, it is better established than the other known iodometric methods, and on the other because the chief reducing substance of the tissues is reduced glutathione. Starting from these considerations, it seemed to us that if there was any shift, it would reflect chiefly a change which could be attributed to glutathione. CoA was separated by the method of Lipmann and Kaplan [15] and its activity was determined colorimetrically according to Bratton and Marachall [16].

RESULTS OF THE INVESTIGATION

We began the investigation with a study of CoA activity in the tissues of irradiated and control animals.

TABLE 1

Change in CoA Activity in Tissues of Rats Irradiated with X-ray
(activity of CoA, in mg acetylated sulfanilamide per 1 g of tissue)

Normal rats		Irradiated rats *			
		after 24 hrs		after 48 hrs	
Liver	Brain	liver	brain	liver	brain
249,0	130,0	150,0	25,0	147,0	52,0
220,0	90,0	138,0	39,0	100,0	128,0
249,0	120,0	165,0	0,00	148,0	104,0
273,0	158,0	90,0	0,00	108,0	69,0
189,0	59,0	110,0	15,0	100,0	126,0
229,0	79,0	25,0	52,0	123,0	49,0
230,0	55,0	57,0	—	199,0	90,0
Average 234,0	99,0	105,0	22,2	132,0	88,3
% Change		—56	—77	—44	—11

* The dose of irradiation in all cases given in this and the following tables was 1200 r.

In Table 1 we give the results of experiments obtained in studying the effect of x-rays on CoA activity in rat tissues. As these results show, the activity of CoA in liver and brain tissues 24 hours after irradiation is definitely lowered; in the brain the decrease reaches 77%. In 48 hours after irradiation, this decrease is less marked, especially in the brain.

In spite of considerable variation in CoA activity in brain tissue of healthy rats, the change resulting from irradiation considerably surpasses the variation found in the unirradiated animals.

TABLE 2

Change in Glutathione Content in Tissues of Rats Irradiated by X-rays
(amount of glutathione in mg %)

Normal rats		Irradiated rats			
		after 24 hrs		after 48 hrs	
liver	brain	liver	brain	liver	brain
225,0	141,0	327,0	150,0	306,0	137,0
264,0	163,0	184,0	134,0	185,0	163,0
244,0	168,0	305,0	173,0	305,0	184,0
268,0	148,0	377,0	163,0	163,0	158,0
254,0	142,0	352,0	158,0	271,0	138,0
222,0	168,0	341,0	190,0	224,0	193,0
208,0	153,0	352,0	147,0	163,0	102,0
204,0	143,0	—	—	273,0	
Average 238,0	153,0	320,0	159,3	236,0	153,0
% Change		+34	± 4	± 0	± 0

As to the change in content of reduced glutathione, as Table 2 shows, 24 hours after irradiation its content in the liver is increased on the average by 34% and after 48 hours its amount has again returned to normal. In the brain of irradiated animals the glutathione content during the whole experimental period remains within normal limits, since the variation found in 24 hours does not exceed the experimental error.

In the next series of experiments we studied the change in sensitivity of CoA to the action of thiol poisons under the influence of irradiation. In other words, we studied the change in reactivity of the SH groups of its molecule under the influence of irradiation. For this purpose, part of the sample in which the CoA activity was determined was treated with a thiol poison which contained arsenic (final concentration $5.0 \cdot 10^{-3}$ M). As Table 3 shows, the sensitivity of CoA to thiol poisons in the liver 24 hours after irradiation remains within normal limits (51-53% inhibition), but after 48 hours it is considerably raised, and the inhibition of activity of CoA at the same concentrations of poison reaches 70%. In the brains of experimental rats in the first 48 hours after irradiation, the CoA under the influence of poisons is almost completely inactivated, that is, its sensitivity to the poison is sharply increased.

TABLE 3

Change in CoA Activity in Rat Tissues Under the Influence of Thiol Poison

(change in activity in %, original activity 100%)

Normal rats		Irradiated rats			
		after 24 hrs		after 48 hrs	
liver	brain	liver	brain	liver	brain
52,0	30,0	24,0	100,0	100,0	100,0
31,0	83,0	100,0	100,0	100,0	100,0
20,0	45,0	83,0	100,0	50,0	100,0
85,0	75,0	45,0	49,0	100,0	75,0
85,0	—	40,0	100,0	100,0	38,0
30,0	28,0	30,0	—	80,0	—
56,0	—	—	—	82,0	—
Average 51,3	52,2	53,0	90,0	87,5	82,6
% Change		+ 3	+72	+70	+58

Thus, as a result of irradiation the sensitivity of CoA to thiol poisons in the periods measured after irradiation shows an increase in the tissues of the irradiated animals compared to the normals (in the liver after 48 hours, in the brain after 24-48 hours).

DISCUSSION OF RESULTS

The present report is a continuation of our work whose results indicate the following. As a result of total irradiation of rats, in definite periods after irradiation we find an increased activity of some tissue thioenzymes and an increase in their sensitivity to poisons. We consider that these facts indicate an increased reactivity of the SH groups of these enzymes. An analysis of the experimental data calls attention to the definite similarity between the results of this and the previous study. Thus, the strong decrease in CoA activity in liver and brain found in the first 24 hours after irradiation gradually lessens. We found an entirely analogous picture in work with brain dehydrogenases [6]. We should remember that the absence of the acetylation process may be due not only to CoA but also to the acetylating enzyme, and the possibility is not excluded that the changes which we observed are connected also with action on the activity of the latter. Our previous findings are also confirmed to a considerable degree in the study of the change in reactivity of the SH groups of CoA. During a definite interval after irradiation the CoA activity of liver and brain under the influence of thiol poisons falls more sharply than in the normals which indicates a change in the properties of the SH groups in the molecule of this compound. It should be said that activation of the thiol enzymes after irradiation in

experiments in vivo has been found by many investigators, but the mechanism has been treated differently. Thus, Feinstein and Bollin [17] explained the activation of carboxypolypeptidase and cathepsin in the intestinal mucosa of irradiated mice, rats, and rabbits by the destruction of a specific inhibitor. The activation of alkaline phosphatase and adenosine triphosphatase in rat spleens was ascribed by Aschwell and Hickmann [18, 19] to involution of this organ. We have given a different explanation of the activation of some enzyme systems in total irradiation of animals. If we consider the simultaneous change in sensitivity of these enzymes and also the absence of a decreased amount of SH groups in the proteins [6] and in the glutathione content (data of the present work), then it seems to us that we have the right to treat these facts as changes in the properties of the SH groups themselves, in protein and nonprotein compounds, and that the changes consist of their increased reactivity. Barron et al. [20] have showed that very slight changes in the molecule of such low molecular weight compounds as the synthetic dithiols are accompanied by considerable changes in the rate of oxidation of the SH groups in these molecules.

The rate of reaction of the oxygen of the air with the SH groups of the dithiols is certainly connected with their reactivity. In its turn, CoA is a sufficiently complex compound and though in comparison with the proteins it has a low molecular weight, we can consider that penetrating radiation will cause some structural changes in its molecule (uniting or splitting some chemical groups, or at least an intramolecular rearrangement), which are accompanied by definite changes in the reactivity of the SH groups which have a catalytic function in this compound.

SUMMARY

CoA activity in rat liver and brain in definite intervals after total irradiation decreases considerably compared to the normals.

CoA activity in liver and brain of irradiated rats is more strongly inhibited by the action of thiol poisons than in the controls. The amount of glutathione in the liver of irradiated rats after 24 hours is somewhat increased. In all the intervals studied it is not changed in the brain.

LITERATURE CITED

[1] M. Serayderian, A. Cohen, and H. Sable, Am. J. Physiol. 177, 150 (1954).

[2] L. M. Kuzin and E. Budilova, Doklady Akad. Nauk SSSR 98, 968 (1954).

[3] D. Kamezin and M. Evera, Biochim. biophys. acta 14, 62 (1954).

[4] G. Barron and J. Dickman, J. Gen. Physiol. 32, 537 (1949).

[5] G. Barron, Radiobiology [Translated into Russian] (IL, Moscow, 1955), p. 249.

[6] M. B. Gintsburg, E. M. Pandre and N. M. Binus, Biokhimiia 22, 467 (1957).

[7] H. Patt, K. Straube, M. Blackford, and D. Smuth, Am. J. Physiol. 167, 7010 (1950).

[8] D. Ia. Graevskii and L. I. Korgan, Doklady Akad. Nauk SSSR 102, 939 (1955).

[9] R. Van-Hemyngen, A. Pirie and J. Boag, Biochem. J. 56, 372 (1954).

[10] H. Du Bois, G. Cottger and D. Peterson, Radiation Res. 2, 79 (1955).

[11] Y. Thomas and F. Micuta, Proc. Soc. Exp. Biol. and Med. 86, 487 (1954).

[12] E. F. Rumiantsev and Z. I. Shuvalova, Biokhimiia 21, 663 (1956).

[13] Z. Bacq and A. Herve, Arch. internat. physiol. 61, 434 (1953).

[14] Wu. Quensel and K. Wachguider, Z. Phys. Chem. 231, 72 (1935).

[15] F. Lipmann and N. Kaplan, J. Biol. Chem. 174, 37 (1948).

[16] A. Bratton and J. Marachall, J. Biol. Chem. 128, 537 (1943).

[17] R. Feinstein and J. Bollin, Proc. Soc. Exptl. Biol. and Med. 83, 6 (1954).

[18] G. Aschwell and J. Hickmann, Proc. Soc. Exptl. Biol. and Med. 80, 407 (1954).

[19] G. Aschwell and J. Hickmann, J. Biol. Chem. 201, 654 (1953).

[20] G. Barron, Z. Mullar, and G. Kalnitsky, Biochem. J. 41, 62 (1947).

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A COLORIMETRIC MICROMETHOD FOR THE DETERMINATION OF CALCIUM IN BIOLOGICAL MATERIAL

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The methods for the determination of calcium in biological material known at the present time are mostly based on the precipitation of calcium as the oxalate with various modifications of the further treatment.

The calcium content is calculated either by weight of the ignited residue (CaO or $CaCO_3$), or by the amount of oxalic acid which is titrated by potassium permanganate [1] or cerium sulfate [2]. Among other methods, calcium oxalate is converted to the carbonate with later titration by acid [3], or the amount of carbon dioxide given off is measured manometrically in a Van Slyke apparatus [4]. A colorimetric method for calcium determination has also been suggested from the content of phosphorus after precipitation of calcium phosphate [5]. Recently a volumetric method for determination of calcium has been worked out based on titration with ethylenediamine tetraacetic acid [6, 7]. However, it should be said that these methods give unreliable results in the determination of microquantities of calcium. Incomplete precipitation of calcium as the oxalate has been recognized by many investigators and is a fundamental difficulty with this method. Also, precipitation as the oxalate requires a long time (from 3-12 hours). Although the method of complex formation is slightly simpler and faster it gives large errors with microquantities of calcium in the object studied, and requires the absence of other alkaline earth elements and phosphorus.

The method suggested by Harrison and Raymond [8] is much more accurate, and is based on precipitation of calcium as the molybdate with later colorimetric determination of the molybdenum by the thiocyanate method. A survey of the work on analytical use of calcium molybdate has been given in the work of Ievins and Grinstein [9].

Harrison and Raymond used the molybdate method for the determination of microquantities of calcium. Their use of a pyridine buffer in which calcium molybdate had a minimum solubility permitted them to precipitate calcium in amounts of 2 μg or more in a volume of 2-3 ml. According to this method the molybdate was then transferred to solution and the molybdenum determined quantitatively; from this the amount of calcium was calculated.

The weakness of this very promising method is the difficulty connected with the determination of microgram amounts of molybdenum by the thiocyanate method. Another difficulty in this method of determining molybdenum is the low stability of the extract and the great sensitivity of the reaction of formation of the thiocyanate complex of molybdenum at the temperature used.

In this connection the thiooxin (8-mercaptoquinoline) which we studied earlier was suggested for colorimetric determination of molybdenum [10], and we decided to use it for indirect colorimetric determination of calcium. The advantages of the thiooxin method for determining molybdenum are the great stability of the molybdenum thiooxinate $MoO_2 \cdot (C_9H_6NS)_2 \cdot H_2O$, its easy extraction by most organic solvents, and the greater stability of the extracts of molybdenum thiooxinate. The sensitivity of the thiooxinate method is not inferior to the thiocyanate method. It permits determination of calcium in amounts from 2 to 50 μg and over.

We used the method for determination of calcium in serum and bone. In particular this method can be recommended for determination of Ca in microquantities of serum (0.1-0.2 ml) as must often be done both in clinical and investigative work.

In the determination of calcium in bones after incineration we observed that in some cases calcium molybdate (precipitated by molybdate-pyridine reagent) came down in the form of large crystals which adhered very slightly to the walls of the centrifuge tube and were easily washed out by the wash liquor which resulted in very low results. We found that addition to the solution being analyzed of one drop of 0.005% solution of soluble starch prevented the formation of large crystals of calcium molybdate and, acting as a binding agent, led to formation of a dense precipitate which did not wash off.

Course of the Analysis

Reagents

1. Thiooxin. 0.5 g of thiooxinhydrochloride [10, 11] is dissolved in 1.7 ml of concentrated HCl and distilled water is added to a volume of 100 ml; the solution is stable for a month.

2. Molybdate-pyridine reagent. A 5% solution of sodium molybdate (C. P.) in 50% ethyl alcohol is allowed to stand for a day and the precipitate of calcium molybdate is filtered off. 75 ml of this calcium-free solution is mixed with 25 ml of pyridine.

3. 2.5 N Hydrochloric acid solution. 20 ml of concentrated hydrochloric acid (sp. gr. 1.193) is mixed with 80 ml of water which contains 5 g of potassium hypophosphite. The potassium (or sodium) hypophosphite is necessary for the formation of yellow, more stable molybdenum thiooxinate.

4. Standard calcium solution. 0.0499 g of calcium carbonate (C. P., dried at 120°) is dissolved with precautions against spattering in 10 ml of 5% HCl, evaporated to dryness on the water bath, dissolved in 20-30 ml of water, and diluted in a calibrated flask to a volume of 100 ml. This gives a solution which contains 20 μg Ca per ml.

5. Xylene or toluene.

6. Starch. A 0.005% solution of soluble starch is prepared in the usual way but without a preservative.

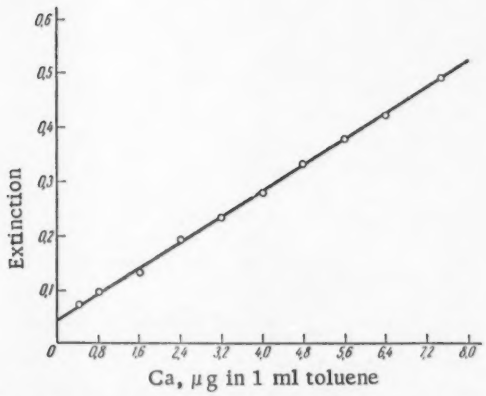

Fig. Calibration curve for determination of calcium by the thiooxin-molybdate method. Extracting agent toluene, measured on a Pulfrich photometer with S-47 filter, cuvette 0.5 cm.

TABLE 1
Determination of Calcium in Pure Solutions by the Thiooxin-Molybdate Method

Ca in μg		Percent error
taken	found	
2	2,2	10
4	3,9	—2,5
8	7,9	—2,3
20	20,3	1,5
30	30	0
40	41,4	3,5
50	51	2

After ignition of the sample and solution of the ash in 2 N HCl, the solution is diluted with water so that the concentration of calcium lies in the limits 4-25 μg per ml, and an aliquot portion of the solution is taken (not more than 2 ml) which contains from 2 to 50 μg of calcium. If the resulting calcium solution has a very acid reaction, it is neutralized with 1-2 drops of ammonia (1 : 3) to a pH of 5-6. In this portion of the solution in a centrifuge tube is added 1 drop of the starch solution and 2 ml of the molybdate-pyridine reagent, and the mixture is kept on the water bath for 30 min at 70°. After cooling to room temperature, it is centrifuged for 10 min at 2500 rpm. The liquid is carefully poured from the centrifuge tube and the edge of the tube is wiped with filter paper. The residual precipitate on the bottom of the tube is washed 5-6 times with 50% alcohol in portions of 1 ml, carefully poured down the walls of the tube. The precipitate is dissolved in 2.5 ml of 2.5 N HCl and the solution is

poured into a separatory funnel; the centrifuge tube is washed three times with the same volume of 2.5 N HCl: 0.5 ml of thiooxin solution is added to the separatory funnel and the molybdenum thiooxinate is extracted by strong shaking with 5 ml of toluene or xylene for 1-1.5 min. The extract is transferred to a 1 cm cuvette (or a 0.5 cm cuvette if the quantity of calcium is more than 50 μg), placed in the photometer, and from the calibration curve constructed in analogous experiments, the desired amount of calcium is found. The toluene or xylene extracts are stable at room temperature for two days. The calibration curve is calculated from Beer's law (Fig. 1).

In Table 1 we give the results of the determination of calcium in pure solutions.

Determination of Calcium in Serum

The chief advantage of this method of determining calcium is the possibility of using it for determination of Ca in microamounts of serum (0.1-0.2 ml), which is of great value in clinical practice and in research, since the amount of serum in many cases is limited.

The method also has interest for the determination of the ultrafilterable calcium of serum, small amounts of which are difficult to determine by other methods.

TABLE 2

Determination of Ca By Oxalate and Thiooxin–Molybdate Methods in Hen Serum, Bone, and Standard Solution

Standard solution					Bone (defatted, dried)		Blood serum of chicken	
Ca taken, in mg	Ca, found by oxalate method in mg	% error	Ca found by thiooxin-molybdate method in mg	% error	found, in %		found, in %	
					by oxalate method	by thiooxin-molybdate method	by oxalate method	by thiooxin molybdate method
1	0,82	—18	1,1	10	19,2	23	25,1	26,3
2	1,78	—11	2	0	23,7	25,3	28,2	29,1
3	2,88	— 4	3,12	4	18	21,1	30	32
4	3,50	—12,5	3,96	—1	18,3	22,8	35	38,4
5	4,75	— 5	5,11	2,2			—	7,2*
								7,8*

* In ultrafiltrate of serum.

The proteins which interfere with calcium determination are usually removed by ignition or by precipitation of the proteins with trichloroacetic acid (TCA). Since the procedure of burning requires considerable time, we have investigated for this method the possibility of using TCA to free the sample from protein. An advantage of TCA also is its ability to transform bound calcium into the ionic state.

To 0.2 ml of serum in a centrifuge tube was added 0.6 ml of twice distilled water and 0.2 ml of 20% TCA solution and the mixture was well stirred. After standing for 10 min, it was centrifuged for 5-7 min; 0.5 ml of liquid was taken from the upper clear layer and transferred to another centrifuge tube; 2 drops of ammonia (1 : 3) were added for partial neutralization of the TCA. The further determination of calcium was carried out as described above.

In the case of a very limited amount of serum, 0.1 ml was taken for analysis, with corresponding decrease in the amount of reagents mentioned above.

Table 2 gives the comparative results of calcium determinations in chicken serum and bone and in standard solutions, by the oxalate and thiooxin-molybdate methods. It shows that the oxalate method gives lower results.

SUMMARY

We have worked out a micromethod for determining calcium by precipitation as the molybdate with later colorimetric determination of the molybdenum by thiooxin. The method permits determination of 2 to 50 μg of Ca in the sample with an accuracy of 5%. The method is particularly effective for determination of Ca in microquantities of serum, which is very important in clinical and research work.

LITERATURE CITED

[1] B. Kramer and F. Tischall, J. Biol. Chem. 47, 473 (1921).

[2] I. Sendroy, J. Biol. Chem. 144, 243 (1942).

[3] A. Sobel and S. Sobel, J. Biol. Chem. 129, 721 (1939).

[4] D. Van Slyke and J. Sendroy, J. Biol. Chem. 84, 217 (1929).

[5] W. Hoffman, Photelometric Clinical Chemistry (New York, 1941).

[6] R. Prshibil, Complexones in Chemical Analysis (IL, Moscow, 1955).*

[7] H. C. Leifheit, J. Lab. and Clin. Med. 47, 623 (1956).

[8] G. E. Harrison and W. H. A. Raymond, Analyst 78, 528 (1953).

[9] A. Ievinš, and V. Grinsteins, Z. analyt. Chem. 127, 20 (1943).

[10] J. A. Bankovsky, Latvijas PSR zinatnu Akademijas Vestis 12, 111 (1952).

[11] Iu. A. Bankovskii, A. F. Levinš, and E. N. Luksha, Zhur. Obshchei Khim. 28, 269 (1958).

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* In Russian.

THE CHANGE IN LIPID METABOLISM IN PLANTS UNDER THE INFLUENCE OF IONIZING RADIATION

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The explanation of the initial changes in plant and animal metabolism under the influence of ionizing radiation has been the subject of study by many investigators [1-12].

For instance, it is known that under the influence of irradiation there is an intermediate stage of activation of a number of processes in metabolism: biosynthesis of proteins, carbohydrates, and nucleic acids [13, 14]. In fats and unsaturated acids under the influence of radiation there may be a chain process of formation of peroxides and hydroperoxides which may lead to an accumulation of peroxides beyond that expected from the dose of irradiation, and because of their toxicity, to a clear biological effect [3-7]. It was shown that in the irradiation of animals the lipids and the enzyme systems which oxidize unsaturated fatty acids are very important in the formation of toxic substances which appear after irradiation [15, 16]. The change in sensitivity of different enzymes in experiments in vivo under the influence of ionizing radiation also were shown by a number of investigators in the case of invertase [13], phosphoglucomutase [17], phosphatases of rat and mouse spleen [15], proteolytic enzymes [18], ribonuclease [13, 14], oxidase [19], spleen phosphorylase [16], alkaline phosphatase [20], adenosine triphosphatase [21], catalase [22], and others. The data indicate a great sensitivity of enzyme systems in vivo because of disruption of total order of biochemical processes [13, 23] and changes in the internal structure of cells after irradiation; these make up the primary mechanism of the biological action of radiation [8].

As our previous experiments showed [11, 24], in studying the influence of radiation on lipoxidase and the substrates of lipid metabolism (unsaturated fatty acids and carotene), in experiments in vitro irradiation of concentrated solutions of enzyme with doses up to 100,000 r actually did not change the activity of lipoxidase from soy seeds. Some inactivation was found only after 48 hours. Only when diluted (5-7 times) starting solutions of lipoxidase were irradiated with the same dose was there noted a fall in activity by 13% in the first hour after irradiation. Thus, it was shown that in experiments in vitro, lipoxidase was very stable to the action of irradiation [11, 24].

Lipoxidases are widely distributed in the plant kingdom. They are found not only in soy beans where they were first discovered [25], but also in different green leaves [26-28], and the seeds of a number of plants [29]. Studies of the lipoxidase activity of plastid-chloroplast and leucoplasts of sugar-beet leaves and the chromoplasts of carrot roots carried out by Sisakian and Kobiakova [30] showed that the plastids have high lipoxidase activity.

Lipoxidase is an autooxidizing enzyme, as the studies of Mikhlin and Pshenova [31, 32] have shown; under the influence of ionizing radiation it can give intense formation of toxic, labile peroxides of unsaturated fatty acids.

In the present work we study the action of x-rays on lipoxidase in experiments in vivo.

EXPERIMENTAL

As objects of study we used the leaves of sprouts of "Primorskaia 592" soy plants, "Moscow" wheat, "Pobeditel" and "Chaika" peas, "Latviia" and "Nemchinovka" beans, dent corn, and "Viner" barley.

We irradiated 10-14 day old sprouts grown in water cultures under laboratory conditions on in the hothouse. By 10-14 days we had obtained fully formed green sprouts, which were then irradiated. In the plants grown under hothouse conditions (beans, soy), the roots of the sprouts before irradiation were carefully washed free of soil, and the plant was then placed in a beaker with water. The plants grown in water cultures were irradiated in Petri dishes. In all cases irradiation took place at a distance of 18-20 cm, and the diameter of the leaf top did not exceed 9-11 cm; for the source of radiation we used an x-ray tube RUP-1 (15 ma, 190 kv). The intensity of irradiation as a rule was 500-700rpm, except for the soy sprouts which were irradiated at an intensity of 1000 rpm.

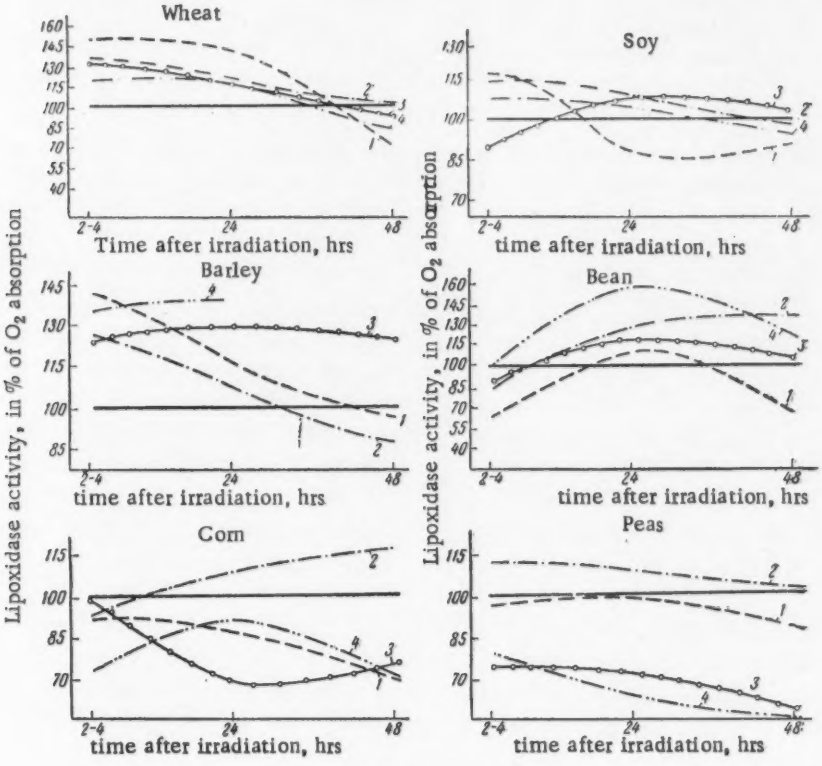

Figure. Effect of irradiation on change in activity of lipoxidase in leaves of the sprouts of different plants.
Dosage of radiation: 1) 1000 r; 2) 10,000 r; 3) 30,000 r; 4) 50,000 r; solid line) control.

Lipoxidase activity in the leaves of the irradiated plants was determined 2, 4, 24, and 48 hours after irradiation. A water extract of lipoxidase from the leaves was obtained as follows: 1-2 g of leaves were ground with quartz sand in 3-5 ml of a phosphate buffer, pH 6.5, then 15-20 ml of distilled water was added. Extraction lasted 20-30 min at room temperature and then the leaf homogenate was centrifuged 5-7 min at 3000 rpm. The resulting clear centrifugate was used to determine the lipoxidase activity. Lipoxidase activity was determined by a manometric method based on measuring the amount of oxygen absorbed during the oxidation of a substrate of sodium linoleate by lipoxidase. Linoleic acid was obtained by the method described in our earlier publications [11, 26].

The determination of lipoxidase activity was carried out as follows. 2 ml of the water extract of lipoxidase from the sprout leaves was mixed in the manometric vessel with 1 ml of the sodium linoleate, prepared immediately before the experiment. As a rule the experiment lasted 60 min at a temperature of 26.1 to 26.6°.

By this method we established the relation of lipoxidase activity in the leaves from sprouts of different plants to the dosage of x-rays. The lipoxidase activity in the leaves of unirradiated plants was determined at the same time as the determination of lipoxidase in irradiated sprouts, and was taken as 100%.

The results of the experiments are given in the figure.

We should remark that the parallel determinations carried out in each series of experiments gave good agreement, with variations not exceeding 2-3%. The variations in the average data for all the experiments can be explained only by variations in the dosimeter during irradiation and by differences in the physiological state of the plants.

It can be seen from the figure that irradiation of corn sprouts with doses from 1000 to 50,000 r lead even after 2-4 hours to a small drop in lipoxidase activity, and then (after 24-48 hours) the activity falls sharply. On the contrary, the lipoxidase of sprouts of wheat, barley, beans, soy, and peas in "activated" in irradiation by the same doses in the first 24 hours after irradiation (that is, the activity in most experiments is above 100%). As shown above, analogous results were obtained in the work with other enzymes [13-22].

The results characterize the action of radiation on lipoxidase in vivo. If in the experiments in vitro [11, 24] even at doses of 100,000 r no change was found in the activity of lipoxidase or there was only a slight lowering of its activity, then when the experiments were run in vivo we could show a change at a much lower dosage, which is also characteristic for the presence of a stage of activation of the enzyme system at relatively low doses and short duration after irradiation; this is well illustrated by the figure and also by the data given in [33].

The considerable change in lipoxidase activity in plants shown even at a dose of 1000 r cannot be explained by a direct change in the enzyme in the irradiation. The actual form of the change in enzyme activity varies for different types of plants. For example, for corn leaves in most experiments there is found only a decrease in lipoxidase activity. Irradiation with high doses (above 30,000-50,000 r) with lengthened incubation beyond 48 hours and also incubation for 72, 96, and 144 hours caused inactivation of lipoxidase in most types of plants. After 72-96 hours incubation the activity of lipoxidase in pea sprouts, for example, fell, depending on the dosage, by 30-50% and in the soy leaves, by 15-20%.

To explain the possible mechanism of increased lipoxidase activity we studied the change in permeability of the leaf tissues after irradiation, since increased permeability might be connected with an increase in the amount of enzyme which was active [8, 13, 14, 23]. The work of Butenko on increase in permeability showed an increased output of electrolytes after irradiation of the rootlets of 3-day old oat sprouts [34]. Analogous data were obtained in work on potato discs [35, 36].

We carried out the determination of the permeability of leaf plasma for electrolytes under the influence of radiation by the method published in the work of Maksimov and Soikina [37]. A weight of leaf of the plant being studied equal to 1 g was put in a beaker and 10 ml of twice distilled water was added. To make exosmosis of electrolytes easy, the leaf was submitted to vacuum infiltration for 1 hour. Then the water was poured off and its conductivity determined. The value of the exosmosis from the leaves of unirradiated plants was taken as 100%.

The data of Table 1 show that, depending on the radiation dosage (1000-50,000 r) there is an increase in permeability of leaf tissue by 5-45%. Measurement of conductivity allowed us to establish a change in over-all "permeability" even at a dosage of 1000 r. The relation between change in lipoxidase activity in leaves of irradiated plants and change in relative permeability on irradiation is certain and indicates that during irradiation there is destruction of the internal cell structure which makes washing the electrolytes out into the external medium easier. From the figure and Table 1 it is evident that increased lipoxidase activity in the experiments in vivo occurs in an analogous way to the change in relative permeability of the tissue. Probably the same change in internal structure or permeability of the cells explains also the observed activation of lipoxidase which thus depends on damage to the internal cell structure during irradiation.

Finally, we studied the effect of irradiation on change in free and bound lipids in the plant leaves. Isolation of the lipids was carried out by extraction with petroleum ether at room temperature according to some modifications of our method described by Zirm, Pongratz, and Polesofsky [38].

TABLE 1

Relative Permeability of Leaf Tissue of Irradiated Plants*

Relative Permeability in Percentage of the Permeability of Unirradiated Plants

Plant	Dosage in thousand r	Time after irradiation in hours		
		2-4	24	48
Bean	1	111,0±2,0	107,0±6,3	109,0±0,5
	10	102,5±0,5	108,5±4,5	105,0±1,0
	30	106,5±1,5	125,0±2,0	126,1±7,0
	50	123,0±0,0	112,0±0,0	147,0±0,0
Pea	10	101,0±16,0	104,0±11,0	116,6±13,3
	30	109,6±16,0	106,5± 5,0	—
	50	133,0± 0,0	128,0± 0,0	149,0± 0,0
Wheat	1	105,6±1,5	100,0± 0,0	—
	10	109,0±4,0	110,7±10,0	102,0±0,0
	30	116,0±0,0	113,0± 0,0	—
Barley	1	109,3±5,8	107,6±1,8	—
	10	106,0±2,0	105,0±5,0	110,0±0,0
	30	103,0±0,0	101,0±0,0	104,0±0,0

* We give averages of two series of experiments carried out with 3-4 parallel determinations.

Irradiated and control leaves from the sprouts of different plants were dried in a vacuum at 3° for 10-12 hours, ground, and a weight of about 1 g was extracted with petroleum ether in a special extractor. The extract was dried over Na_2SO_4 and the petroleum ether was evaporated in a stream of nitrogen. The residue was dried over NaOH, dissolved in ether, 10 ml of 5% NaOH was added, the ether was evaporated off on the water bath and the residue heated for 5 hours. The nonsaponified part was removed with ether, the residue was acidified with dilute hydrochloric acid, heated briefly, and after cooling was extracted with ether and the insoluble part separated. After drying the ether solution over Na_2SO_4 it was evaporated and in the residue which consisted of free lipids we determined the amount of peroxides formed in irradiating the plants.

The bound lipids were obtained from the leaf residues after extraction with petroleum ether by their treatment with 50 ml of 80% alcohol and 5 hours heating under reflux. After the solution was cooled, the alcohol was distilled off and the residue was extracted with petroleum ether. The petroleum ether was washed free of alcohol with water and dried; it was then treated as described above for the free lipids.

Peroxides were determined in the free and bound lipids of control and irradiated plants by the method of Glavind and Hartmann [39]. For this purpose the residue of free or bound lipid was dissolved in 5 ml of a mixture consisting of equal parts of chloroform and absolute alcohol and 2 drops of "leuco reagent" were added. For the preparation of the latter, 2,6-dichlorophenolindophenol was dissolved in hot distilled water, filtered and reduced with ascorbic acid. The precipitate of dichlorohydroxyphenylamine ("leuco") was redissolved in absolute alcohol, impurities were removed by activated charcoal, and it was precipitated by adding twice distilled water, washed carefully, and dried in a vacuum over silica gel.

After N_2 had been passed for 10 min into the reaction mixture prepared for the determination of peroxides, we added one drop of ferric chloride solution (100 mg of $FeCl_3$ in 100 ml of absolute alcohol, and after 10 min compared the extinction with the control in a spectrophotometer at 530 mμ.

The results in Table 2 show that a sharp change is found even at a dose of 1000 r. It also follows from these results that in the fraction of free lipids from leaves of irradiated sprouts there are five to six times more peroxides than in unirradiated leaves. These results, the first shown for plants, considerably surpass those obtained

earlier for animals [3-7]. The observed oxidation probably depends on the action of enzymes in vivo, since the irradiation of substrates of lipid metabolism in vitro [11, 24] gives no such effect.

Thus, the characteristic feature of the action of radiation on the lipoxidase system in vivo is the occurrence of change at lower dosages than in vitro and the presence of a stage of activation of enzyme systems at a rather low dosage and a short time after irradiation.

TABLE 2

Effect of Ionizing Radiation on Change in Composition of Free and Bound Lipids in Leaves of 14-Day-old Sprouts of Plants

Plant studied	Dosage of radiation, in r	Free lipids		Bound lipids	
		Amount, in g	Amt. of peroxides in milleequivalents per kg dry weight	Amount, in g	Amt. of peroxides, in milleequivalents per kg dry weight
Bean	Unirradiated	0.02	0.411	0.02	0.1615
	1000	0.03	2.30	0.02	0.1658
Barley	Unirradiated	–	16.60	–	0.7954
	1000	–	83.80	–	0.8390

• We give averages of two parallel determinations 24 hours after irradiation for bean and 6 hours after irradiation for barley.

The observation that in the experiments in vitro even at dosages of 100,000 r there was no change in lipoxidase activity or a small decrease in it shows that the considerable changes produced in enzyme activity in vivo even at dosages of 1000 r cannot be explained by a direct change of lipoxidase in irradiation.

The change in relative permeability in the leaves of plant sprouts under the influence of ionizing radiation (Table 1) shows that in irradiation there is damage to the internal structure of the cells which makes it easy to wash out the electrolytes into the outer medium.

Probably this change in internal structure or permeability of the cell can explain the observation of change in lipoxidase activity which thus depends on a secondary change in cell structure during irradiation.

The presence of an intermediate stage of activation of a number of metabolic processes (biosynthesis of proteins, carbohydrates, nucleic acids, etc.) in plants during irradiation was also shown in the dissertation of Kalacheva [14]. The general significance of this effect in connection with the theory of radiobiological action was considered earlier by one of us [8].

The actual nature of the change in internal structure of the cells could vary for different types of plants; thus, for example, the leaves of corn in most experiments showed only slight decrease in activity.

Irradiation with high dosages (above 30,000-50,000 r) with incubation prolonged beyond 48 hours caused inactivation of the lipoxidase in most of the types of plants studied.

The considerable increase in amount of peroxides of unsaturated fatty acids (Table 2) even at low dosages of irradiation (1000 r) was interesting.

In view of the considerable physiological action and toxic properties of the peroxides of unsaturated fatty acids [40] the study of the conditions of their formation should have great value.

SUMMARY

We have studied the action of ionizing radiation on lipoxidase activity in the sprouts of different plants. The results, the first given for plants, indicate a considerable change in the lipoxidase enzyme systems in vivo under the action of x-rays.

We have shown a very great sensitivity of lipoxidase in plant leaves in vivo to the action of ionizing radiation. We have observed a change in lipoxidase activity at much lower dosages than in the experiments in vitro, by 30-50%, and the presence of a stage of activation of the enzyme systems, at comparatively low dosages of radiation and short periods of incubation after irradiation.

We have established the existence of a change in relative permeability in the leaves of plant sprouts under the action of ionizing radiation which indicates a destruction of internal cell structures leading to facilitation of the washing of electrolytes out into the outer medium. The change in total permeability or the change in internal structure probably leads to the observed change in lipoxidase activity when the plant sprouts are irradiated.

We have observed a considerable increase in the amount of peroxides of unsaturated fatty acids at low dosages of radiation (1000 r) in the leaves of bean and barley sprouts which probably depends on the action of enzymes.

We can assume that the considerable physiological action and toxic properties of the peroxides of unsaturated fatty acids plays a great part in the intoxication of plant organisms under irradiation.

LITERATURE CITED

[1] B. Jolles, Nature 164, 163 (1949).

[2] G. Strelin, Doklady Akad. Nauk SSSR 73, 1283 (1950).

[3] P. Edelmann, Feder. Proc. 14, 131 (1955).

[4] G. P. Gruzdev, Papers and Addresses, All-Union Conference on Medical Radiology, (Medgiz, Moscow, 1956).*

[5] A. S. Mochalina, Papers and Addresses, All-Union Conference on Medical Radiology, (Medgiz, Moscow, 1956).*

[6] B. N. Tarusov, Papers and Addresses, VII All-Union Session on Physiology, Biochemistry, Pharmacology (Izd. AN SSSR, Moscow, 1955), p. 599. *

[7] P. Dobouloz and I. Dumas, Compt. rend. Soc. biol. 148, 705 (1954).

[8] A. G. Pasynskii, Biofizika 2, 566 (1957).

[9] G. S. King, Amer. J. Bot. 36, 256 (1949).

[10] A. M. Kuzin, Papers and Addresses, All-Union Conference on Medical Radiology (Medgiz, Moscow, 1956). *

[11] E. V. Budnitskaia, I. G. Borisova, and A. G. Pasynskii, Doklady Akad. Nauk SSSR 110, 603 (1956).**

[12] R. Holman and S. Bergstrom, Adv. Enzymol. 8, 425 (1948).

[13] N. M. Sisakian, The Action of Radiation on Organisms (Izd. AN SSSR, Moscow, 1955), p. 137. *

[14] V. Ia. Kalacheva, Action of Radiation on Some Metabolic Processes in the Plant Organism, Dissertation (Moscow, 1957). *

[15] Ed. Uvrki, Federation Proceedings 14, 295 (1955).

[16] H. I. Eichel, Proc. Soc. Exptl. Biol. Med. 88, 158 (1955).

[17] N. B. Strazhevskaia, Studies of Some Initial Biochemical Changes in Plants Under the Influence of Ionizing Radiation, Dissertation (Moscow, 1956). *

[18] V. P. Nilova, Tr. po Prikladnoi Botanike, Genetike i Selektsii, Ser III, 14, 109 (1936).

[19] L. Johnsonee, Bot. Gas. 82, 373 (1926).

[20] M. N. Maisel, Action of Radiation on Organisms (Izd. AN SSSR, Moscow, 1955), p. 91.*

* In Russian.
** Original Russian pagination. See C. B. Translation.

[21] Z. M. Bacq, Radiobiolog. Symposium (1954).

[22] R. Feinstein, Radiation Res. 4, 217 (1956).

[23] E. V. Budnitskaia, Usp. Sovr. Biologii 3, 280 (1957).

[24] E. V. Budnitskaia, I. G. Borisova, and A. G. Pasynskii, Biokhimiia 21, 702 (1956).**

[25] E. Andre´ and R. W. Hov, Compt. rend. Soc. biol. 194, 645 (1932).

[26] E. V. Budnitskaia, Biokhimiia 20, 611 (1955).

[27] H. Sŭllman, Experientia 1, 322 (1945).

[28] P. A. Kolesnikov, Doklady Akad. Nauk SSSR 71, 1085 (1950).

[29] V. L. Kretovich and Iu. S. Rall, Tr. Mosk. inst. Pishchevoi Promyshlennosti 1 (1952).

[30] N. M. Sisakian and A. M. Kobiakova, Biokhimiia 22, 516 (1957).**

[31] D. M. Mikhlin, Biological Oxidations (Izd. AN SSSR, Moscow, 1956).*

[32] D. M. Mikhlin and K. V. Pshenova, Biokhimiia 13, 76 (1948).

[33] E. V. Budnitskaia, I. G. Borisova, and A. G. Pasynskii, Doklady Akad. Nauk SSSR 120, 131 (1958).***

[34] R. G. Butenko, Doklady Akad. Nauk SSSR 72, 197 (1950).

[35] O. S. Engel', Doklady Akad. Nauk SSSR 75, 437 (1952).

[36] N. Higinbotham and K. Mika, Plant Physiol. 29, 174, (1954); 30, 103 (1955).

[37] N. A. Maksimov, Collected Works on Drought Resistant and Winter Resistant Characteristics, (Izd. AN SSSR, Moscow, 1952), Vol. 1, p. 478.*

[38] K. L. Zirm, A. Pongratz, and W. Polesofsky, Biochem. Z. 326, 405 (1955).

[39] I. Glavind and S. Hartmann, Acta Chem. Scand. 9, 497 (1955).

[40] V. L. Kretovich and A. A. Bundel', Biokhimiia 10, 216 (1945).

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THE POLYPHENOL OXIDASE ACTIVITY OF GROWING AND PROCESSED TEA SPROUTS

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The polyphenol oxidase (PPO) of tea is an enzyme which activates the basic process of tea production, fermentation. In this connection, a study of this enzyme in tea has been made by a large number of investigators [1-10].

Aside from its great practical value for improving tea production, the study of PPO activity in tea sprouts has great intrinsic interest from the viewpoint of general plant biochemistry. As is known, the tea plant contains a large number of tannins which under certain conditions have an inactivating effect on enzymes. It is not yet clear whether there is absence of enzyme inactivation by tannins in vivo. In the present work we have studied some of the problems of interaction of PPO and tannins of tea and other plants.

The material for the study consisted of tea plants grown in the tea plantations in Anaseuli (Georgian SSR).

According to existing data [2-7, 10], PPO in tea leaves occurs in two forms, free and bound to the plastid cells. Therefore, in the present study we determined the activity of both forms of the enzyme. In all cases the enzyme preparations were obtained by treatment of the material being investigated with cooled acetone to remove tannins and other acetone soluble substances, with later separation of the preparation into water soluble and insoluble fractions [7]. The PPO activity was determined by absorption of oxygen in a Warburg apparatus by the enzyme preparation in the presence of the substrate (pyrocatechin). The determination was carried out at 30° for 2 hours, and as the incubation medium in all the experiments we used 2 ml of 0.33 M phosphate buffer, pH 5.9. The total amount of tannins was determined by titration according to Loewenthal.

In Table 1 we give the results obtained by determining the PPO activity in different parts of the young sprouts which serve as the source of preparation of black and green tea, and also the fully formed leaves and the coarse leaves which can only be used for production of green brick tea [11].

As the results in Table 1 show, the younger the tea leaf, the higher its total activity of PPO and the more of this in soluble form. The great relative content of soluble PPO in the stem indicates the mobility of this form of the enzyme in the plant. The higher content of tannins in young tea leaves is not accompanied by a simultaneous decrease in their PPO activity.

In the studies of Oparin and Kursanov [12] it was shown that the action of very weak concentrations of tannins on enzymes caused inactivation of the enzymes. The most probable explanation why in living or damaged plant tissues the enzymes are not inactivated by tannins is the assumption of Kursanov [13] that the enzyme proteins have a specific resistance to the tannins of the plants which contain the enzyme. Our results confirmed the correctness of this assumption. While the tannin preparations isolated from tea leaves were oxidized by PPO from different sources (Fig. 1), only the tea PPO was stable toward the tea tannins; PPO from potato tubers and the fungus Bolethus scaber were quickly inactivated.

The specific stability of enzyme proteins to the tannins of the corresponding plant evidently arises as an adaptive accommodation during development. Similar indications are given in our experiments on the growth of Aspergillus niger in aqueous extracts of tea leaves. In this case the PPO produced in the mycelia is stable toward tea tannin. In Aspergillus niger grown in a synthetic medium the polyphenol oxidase activity is not seen at all (Fig 2).

According to the data of Mikhlin and Pshenova [14, 15] the inactivation of enzymes by phenolic substances is produced by the products of their oxidation. Our results make this assumption probable also for tannins.

TABLE 1

PPO Activity and Content of Tannins in Portions of Young Sprouts and in Mature and Old Tea Leaves

Source of enzyme	Absorption of O_2 in μ l/hr			Tannins in % of dry weight
	total preparation	soluble fraction	insoluble fraction	
Bud	123	17	106	32,0
1st leaf of sprout	140	28	112	32,8
2nd leaf of sprout	136	22	114	26,0
3rd leaf of sprout	131	15	116	22,5
Stem	130	31	99	12,9
Mature leaf	122	7	115	14,8
Old leaf	82	1	81	9,0

Potato PPO preparations were incubated with water extracts from tea leaves (1 ml of extract contained 2.5 mg of tea tannins) for 4 hours without adding oxygen (in a vacuum), and in the presence of oxygen. After the incubation the enzyme was freed from tannins with cooled acetone and a comparative determination was made of the PPO activity of the incubated preparation and its starting activity.

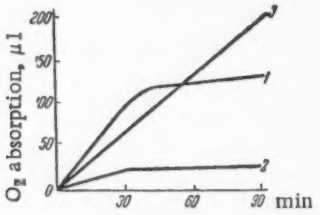

Fig. 1. Oxidation of tea tannins by PPO of tea leaves, potatoes, and the fungus Bolethus scaber.
In the experiment: 10 mg of enzyme preparation; 16 mg of tea tannin preparation. 1) Potato PPO, 2) fungus PPO, 3) tea PPO.

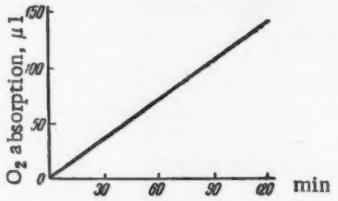

Fig. 2. Oxidation of tea tannins by PPO of Aspergillus niger grown in extract of tea leaves.
In the experiment: 10 mg of enzyme preparation; 8 mg of tea tannin preparation.

The data given in Table 2 show that if the PPO preparation had been in contact with tannins in the absence of oxygen, the enzyme kept its activity almost completely, but when oxygen was admitted and the tannins were oxidized by the PPO enzyme preparation, the enzyme was completely inactivated.

These results, like the data from other experiments, makes it probable that the resistance of the enzyme and other proteins to the tanning action of tannins in live plants depends in considerable measure on the non-oxidized state of these substances.

When the cells of tea leaves are damaged, as a result of a weakening of the reducing processes there is energetic enzymatic oxidation of the tannins. On this, as our experiments showed, there is only a slight decrease in PPO activity and part of the soluble enzyme enters into combination with the oxidized tannin. Since these results have great value for the practical production of tea, we studied the change in PPO activity during tea preparation.

Two-leafed tea sprouts were dried naturally for 18 hours. From the material at different stages in its preparation we obtained enzyme preparations in which we determined the PPO activity.

TABLE 2
Potato PPO Activity After Incubation With Tannins in the Presence of Atmospheric Oxygen and Without Adding Oxygen
In the experiment: 100 mg of enzyme preparation, 3 mg of pyrocatechin

Material	Oxygen absorption, μl/hour
Starting PPO preparation	138
The same after incubation with tannins in a vacuum	132
The same after incubation with tannins with admission of atmospheric oxygen	0

The results in Table 3 indicate that PPO activity in the different stages of tea preparation undergoes a great change. When the tea sprouts are dried there is increased activity in soluble PPO. This effect may be the result of removal of part of the enzyme from the plastid as a result of dehydration of the tea sprouts and the biochemical changes connected with this, as Sisakian and his co-workers [16] found in work with the plastid enzymes of other plants. In the fermentation process, as Table 3 shows, there is a sharp decrease in activity of soluble PPO. The activity of the insoluble fraction is correspondingly increased which indicates the transformation of soluble enzyme into the bound state.

TABLE 3
PPO Activity at Different Stages in the Treatment of Tea
In the experiment: 40 mg of enzyme preparation, 5 mg of pyrocatechin

Material	O_2 Absorption in μl/hour		
	total preparation	soluble fraction	insoluble fraction
Fresh leaves	103	19	84
Dried leaves	124	41	83
Leaves after 1 hr fermentation	120	30	90
Leaves after 3 hrs fermentation	118	6	112

In order to show how the oxidation of the tannins affects the binding of the soluble enzyme, we took two-leaf tea sprouts which had been dried and ground them quickly in a porcelain mortar; then part of the resulting material was fermented in the presence of atmospheric oxygen and another part put under vacuum. We obtained material after 3 hours of fermentation and determined the PPO activity in it.

From the results in Table 4 we can see that while the fermentation of tea with admission of atmospheric oxygen gives a sharp decrease in activity of soluble PPO, in the damaged sprout tissues placed under vacuum there is only a slight decrease in activity. The decreased activity of the soluble enzyme can be explained as the result of the reaction of the oxidation products of the tannins with the enzyme protein with formation of an insoluble complex. There is reason to believe that there is then obtained a stable compound, since the chemistry of this process is obviously analogous to the formation of bound tannins. As is evident from the work of Bokuchava and Popov [17] the bond of the components (tannins + proteins) in bound tannins differs greatly in strength.

We succeeded in showing (Table 5) that the formation of a stable compound with protein did not occur if the tea tannin was present in the unoxidized state. In this case the tannin could easily be washed from the protein by different solvents. When we took the rapidly ground tea leaves and fermented one part of them for

4 hours at 30° with admission of atmospheric oxygen, while another part was kept for the same period in a vacuum; then we showed that in the latter case, in spite of close contact of the tannins with the tea leaf proteins, binding of the tannins did not occur. When atmospheric oxygen was admitted there was obvious formation in the ground material of bound tannins in the fermentation of tea. The determination of bound tannins was carried out by the method of Bokuchava and Popov [17].

TABLE 4

PPO Activity of Ground Tea Leaves in the Presence of Atmospheric Oxygen and When They Are Kept Without Admitting Oxygen
In the experiment: 50 mg of enzyme preparation, 5 mg of pyrocatechin

Source of enzyme	O_2 Absorption in μl/hour		
	total preparation	soluble fraction	insoluble fraction
Dried leaf	126	51	75
The same after 3 hrs fermentation	121	10	111
The same after 3 hours under vacuum	124	45	79

Analogous results were obtained if, instead of tea leaves we took the tannins isolated from tea and oxidized them by a PPO preparation.

Thus, in the light of these results, it is very probable that the decreased activity of the soluble fraction of PPO during fermentation is due to binding of the fermented proteins by the oxidation products of the tannins.

TABLE 5

Amount of Soluble and Bound Tannins in the Fermentation of Ground Tea Leaves in the Presence of Atmospheric Oxygen and When They Are Kept Without Admitting Oxygen

Material	Tannins in ml of 0.1 N $KMnO_4$ per 1 g of dry weight	
	soluble	bound
Ground tea leaves	57.5	2.5
The same after 4 hours fermentation	23.7	10.0
The same after 4 hours keeping in a vacuum	57.5	2.5

All the effects described above have great value for the life of plants which contain tannins. We can think that the unoxidized tannins in the life of the plant cell are less capable of close reactions with protein compounds.

When the cell is damaged, the oxidation products of the tannins which accumulate as a result of intense oxidation by the enzymes, have a greater ability to react with protein or its decomposition products. This may be of significance in the attack on plants by one or another pathogenic organism [18, 19]. It may be that the occurrence in the damaged cell of oxidation products of the tannins may lead to reaction with the proteins of the microorganisms to form stable complexes so that the site of the attack is localized. These facts illustrate the active physiological role of tannins in the life of plants.

SUMMARY

We have studied the activity of polyphenol oxidase in the sprouts of tea plants which are the raw materials for the manufacture of tea, and shown that the younger the leaf, the higher its activity of PPO and the greater the portion of the enzyme found in the soluble form.

The existance of inactivation of the enzyme by the tannins in the plants, aside from the specific resistance of the enzyme proteins, evidently depends also on the ability of the tannins to react closely with the proteins only after their oxidation.

When the tea sprouts are treated to give black tea, there is a marked change in PPO activity. During drying of the sprouts there is increased activity in the soluble form of PPO. During fermentation of the tea there is a slight decrease in PPO activity and much of the soluble enzyme is bound with the oxidation products of the tannins. The enzyme in the bound form does not lose its activity.

We wish to express our thanks to Professor M. A. Bokuchava for very valuable advice in carrying out this work.

LITERATURE CITED

[1] A. I. Oparin and T. A. Shubert, Biochemistry of Tea Production 6, 82 (1950).*

[2] M. A. Bokuchava, T. A. Shubert, and V. R. Popov, Biokhimiia 13, 42 (1948).

[3] M. A. Bokuchava, Tannins and Oxidizing Enzymes of Georgian Tea, Dissertation (Moscow, 1949).*

[4] M. A. Bokuchava and T. A. Shubert, Biochemistry of Tea Production, 6, 90 (1950).*

[5] T. A. Shubert, Phenolase Systems and Their Role in Respiration and Fermentation of Tea, Dissertation (Moscow, 1951).*

[6] V. R. Popov, Oxidation Processes in Tea Production, Dissertation (Moscow, 1952).*

[7] H. Sreerangachar, Biochem. J. 37, 653 (1943).

[8] E. Roberts, and D. Wood, Biochem. J. 47, 175 (1950).

[9] E. Roberts, J. Sci. Food. Agric. 3, 193 (1952).

[10] L. Li and J. Bonner, Biochem. J. 41, 105 (1947).

[11] M. A. Bokuchava, Biochemical Basis of Production of Lao-Cha and Green Brick Tea (Izd. AN SSSR Moscow, 1955).*

[12] A. I. Oparin and A. L. Kursanov, Biochem. Z. 209, 181 (1929).

[13] A. L. Kursanov, Biochemistry of Tea Production 1, 38 (1936).*

[14] D. M. Mikhlin and K. V. Pshenova, Doklady Akad. Nauk SSSR 101, 313 (1955).

[15] D. M. Mikhlin, Biological Oxidations (Izd. AN SSSR, Moscow, 1956).*

[16] N. M. Sisakian, Enzymatic Activity of Protoplasmic Structures (Izd. AN SSSR, Moscow, 1951).*

[17] M. A. Bokuchava and V. R. Popov, Biokhimiia 10, 234 (1945).

[18] T. White, J. Sci. Food Agric. 8, 377 (1957).

[19] B. A. Rubin and V. A. Aksenova, Biokhimiia 22, 202 (1957).

Received February 19, 1958

* In Russian.

THE EFFECT OF THE DIPEPTIDES ANSERINE AND CARNOSINE ON OXIDATIVE PHOSPHORYLATION IN ISOLATED MITOCHONDRIA OF THE PECTORAL MUSCLE OF THE PIGEON

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In previous work from our laboratory we have established [1-7] that when carnosine and especially anserine are added to the ground tissues of pigeon pectoral muscle, the effectiveness of respiration with respect to formation of energy-rich phosphorus compounds is increased. When creatine is used as a phosphate acceptor, the amount of phosphocreatine which is formed in incubation of minced muscle is increased, and the value of the phosphorylation coefficient P:O is raised. It has also been shown that the toxic effect of azide, a poison which separates respiration and phosphorylation, is decreased when anserine is added. These results were obtained with ground skeletal muscle tissue.

The purpose of the present work is to show the effect of these dipeptides and also histidine and β-alanine on the process of oxidative phosphorylation in mitochondria isolated from muscles, since it is known that this process is localized in them.

EXPERIMENTAL METHODS

It was established in preliminary studies that the isolation of mitochondria from homogenates of pigeon pectoral muscle was unsuccessful when fractional centrifuging was carried out in a solution which contained sucrose (0.25 M) and ethylenediamine tetraacetate (EDTA, 0.001 M). When the mitochondria were washed once in this medium, the P:O ratio fell to less than unity, and after two washings, the binding of mineral phosphate became very weak, almost absent, in spite of continued oxygen absorption.

Considerably better results were obtained by the use of the method of Chapell and Perry [8] with minor modifications. We used salt solution I for the separation of the mitochondria. The final concentrations of its ingredients were: KCl 0.176 M, $MgSO_4$ 0.005 M, EDTA 0.001 M, ATP 0.001 M, pH 7.4. The osmotic concentration (0.37 M) was checked by the cryoscopic method. The mitochondria of pigeon pectoral muscle, twice washed in this medium, were characterized by a clearly shown ability for oxidative phosphorylation. The experiments were set up as follows. The pigeons were sacrificed by decapitation, the large pectoral muscle was quickly cut out and roughly cut up with scissors in the cold. The required amount of tissue (about 7-8 g) was weighed out and homogenized in a glass homogenizer with ten times its volume of solution I. The undamaged cell nuclei, myofibrils, etc. were separated by centrifuging for 5 min at 0° and 600 g. The supernatant liquid was poured off and repeatedly centrifuged in the cold for 10 min at 8000 g. After removal of the supernatant liquid, the yellow-brown residue of mitochondria was again suspended in solution I and centrifuged once more at 8000 g. This operation was repeated twice and the residue of washed mitochondria was suspended in solution I in such amount that the volume of mitochondria corresponding to 1 g of tissue was contained in 1 ml of sample. Nitrogen was determined by the Kjehldahl method and the determination showed that in this case 1 ml of sample contained about 2.5 mg of nitrogen.

The experimental samples with a volume of 2 ml were placed in a Warburg flask as follows: to 1.3 ml of a phosphate buffer solution II was added 0.3 ml of a solution of hexokinase obtained according to Berger and purified to stage 3a [9] and 0.4 ml of mitochondria suspension. 1.3 ml of solution II contained: 75 μ mole of a mixture of K_2HPO_4 and KH_2PO_4, 45 μ mole α-ketoglutaric acid; 20 μ mole $MgSO_4$; 6.5 μ mole ATP; 2 μ mole EDTA; 60 μ mole glucose. The pH (7.4) was controlled potentiometrically with a glass electrode. Incubation was carried out in an O_2 atmosphere at 26°. Absorption of oxygen was followed monometrically; the intensity of phosphorylation was estimated by the decrease in mineral phosphate, determined by the method of Sumner [10]. The degree of swelling of the mitochondria was followed with an electronephelometer by the optical density method of Cleland [11], using filter No. 10 (520 mμ). The relative volume of the mitochondria was measured in a hematocrit.

The fermentation process was stopped at the beginning of the experiment and at different periods of incubation by adding 2 ml of 5% trichloroacetic acid.

RESULTS AND DISCUSSION

Several variants of the experiments were carried out.

1. The first series of experiments was run with freshly separated mitochondria which were added to solution II (control sample) and to solution II which contained anserine (experimental sample).

TABLE 1

Oxidative Phosphorylation in Freshly Separated Mitochondria Suspended in Solution II Without Anserine (Control Sample) and With Addition of Anserine (Experimental Sample) (incubation in O_2 atmosphere at 26°; anserine content in 2 ml of experimental sample 50 μ mole; ΔO) absorption of oxygen; ΔP) bound phosphorus).

Date of experiment	Incubation time in min	Control sample			Experimental samples		
		ΔO in μ atoms	ΔP in μ atoms	P:O	ΔO in μ atoms	ΔP in μ atoms	P:O
9.IV 1957	30	15,8	45,2	2,86	17,8	48,6	2,75
10.IV 1957	20	6,5	14,0	2,15	5,5	12,5	2,26
26.IV 1957	30	6,2	15,5	2,5	6,0	15,5	2,58
	60	13,6	23,5	1,73	13,6	23,8	1,75
	120	28,6	24,0	0,84	25,7	24,5	0,95

The results are given in Table 1. The data of Table 1 illustrate the fact that the content of anserine in the incubation samples does not affect absorption of O_2 and binding of mineral phosphate by freshly separated mitochondria, and hence, the P:O coefficient. Increase in the permeability of mitochondria by preliminary standing in water for an hour at room temperature and later incubation in phosphate buffer II with or without addition of anserine does not change the result: the value of P:O in both cases remains the same.

2. In the next series of experiments the mitochondria of pigeon pectoral muscle were isolated in solution I and twice washed in it. A part of the washed mitochondria was suspended again in solution I and the other part in solution I with addition of anserine (solution IA). The final anserine concentration in solution IA was 0.05 M and the KCl concentration was decreased to 0.137 M. The depression of both solutions I and IA was the same and corresponded to an osmotic concentration of 0.37 M. The pH was checked with a glass electrode and came to 7.4. Both mitochondria suspensions (in solutions I and IA) immediately after preparation and also after 20, 40, and 60 min standing ("aging") at room temperature were treated with 0.4 to 1.6 ml of a mixture poured into the Warburg vessel which was composed of 1.3 ml of phosphate buffer (solution II) and 0.3 ml of hexokinase solution.

The results of determination of O_2 absorption and binding of mineral phosphate which were obtained in this series of experiments are given in Table 2.

From the results in Table 2 it is evident that oxidative phosphorylation in mitochondria "aged" in solution I without anserine falls much more sharply than in mitochondria "aged" in solution IA, which contains anserine. In other words, the presence of anserine in the medium when mitochondria are "aged" preserves the relation between respiration and phosphorylation.

TABLE 2

Oxidative Phosphorylation in Mitochondria "Aged" in Solution I (Control Sample) and in Solution IA (Experimental Sample)

After "aging" the mitochondria were incubated in solution II in an O_2 atmosphere for 30 min. at 26°

Date of experiment	"Aging" time in min	Control samples			Experimental samples		
		ΔO in μ atoms	ΔP in μ atoms	P:O	ΔO in μ atoms	ΔP in μ atoms	P:O
20. VIII 1957	0	21,8	52,0	2,38	23,2	57,0	2,45
	20	22,8	48,5	2,14	22,4	51,4	2,30
	40	26,2	19,1	0,73	23,2	45,0	1,93
	60	16,9	2,5	0,16	19,6	9,5	0,49
22. VIII 1957	0	11,5	10,4	0,91	15,7	17,4	1,11
	20	14,0	7,8	0,56	14,7	16,4	1,11
	40	15,0	0,4	0,03	13,2	14,3	1,08
	60	10,9	0	0	13,3	0,8	0,06
28. VIII 1957	0	16,6	30,4	1,84	17,6	42,0	2,4
	20	23,6	14,2	0,6	20,9	39,9	1,9
	40	20,2	10,5	0,52	17,5	35,5	2,0
	60	20,2	7,0	0,35	19,1	34,4	1,8

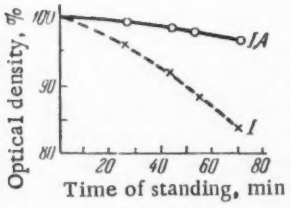

Fig. 1. Change with time in optical density of samples of mitochondria "aged" in solution with anserine and without anserine at 24.5°.
1) Sample without anserine; 1A) sample with anserine; starting density 100%.

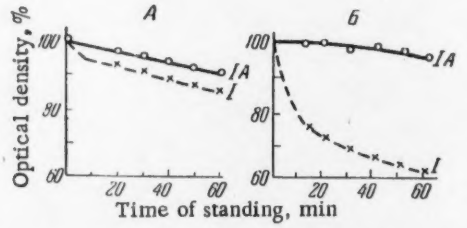

Fig. 2. Change with time in optical density of samples of mitochondria "aged" in solutions without anserine (at different pH) and solutions with anserine at 24°.
A) Experiment of Aug. 22, 1957; I) sample without anserine, pH 7.2 after 60 min; IA) sample with anserine, pH 7.35 after 60 min.
B) Experiment of Aug. 28, 1957; I) sample without anserine, pH 7.2 after 60 min; IA) sample with anserine, pH 7.3 after 60 min; starting density 100%.

3. According to the literature, separation of the processes of respiration and phosphorylation is often connected with change in the structure of the mitochondria – with their swelling [12, 13]. Actually, the increase in volume of the mitochondria was more evident when they stood in solution I than in solution IA. A test of the optical density of the mitochondria when they stood for different periods in solutions I and IA gave results (Fig. 1) which clearly showed the ability of anserine to keep the mitochondria from swelling.

4. It is necessary to emphasize that the pH in the mitochondria suspension after they had stood in solution IA was always 0.05-0.2 higher than when they stood in solution I. This was related to the fact that anserine, as is known, has a weak buffering action. This raised the question of whether the ability of anserine to maintain

the connection between respiration and phosphorylation and also to prevent swelling of the mitochondria depended on the higher level of pH which resulted from the buffering action of the dipeptide. Although there are data in the literature [14] on the absence of morphological change in mitochondria when the reaction medium lies in the pH limits of 5 to 9, we decided to test this question experimentally. In this series of experiments the pH of solution I, in which the mitochondria were suspended, was brought to a more alkaline reaction than pH 7.4, so that after the mitochondria had stood in it for an hour, the pH was not lower than in solution IA. Figure 2 shows the results obtained in experiments on Aug. 22 and Aug. 28, 1957. The data relating to absorption of O_2, fall in mineral phosphate, and P:O coefficient in these experiments are given in Table 2.

In the experiment of Aug. 22, 1957, the pH of the mitochondria suspension after one hour standing in solution I was 7.2, and in solution IA, 7.35. In the experiment of Aug. 28, 1957, the pH of the mitochondria suspension in solution I was 7.6 and in solution IA, 7.3. Thus, although in the experiment of Aug. 22, 1957, after "aging" the suspension of mitochondria in solution IA, had a pH 0.15 higher, and in the experiment of Aug. 28, 1957, 0.3 lower than in the suspension of mitochondria "aged" in solution I, the observed relation was the same: oxidative phosphorylation (Table 2) in both cases was maintained better in the mitochondria "aged" in solution IA than those in solution I.

The change in optical density of the mitochondria in the experiments of Aug. 22 and 28, 1957 was qualitatively in the same direction. However, in the experiments of Aug. 28, 1957 the effect of the action of anserine was great, while in the experiments of Aug. 22, 1957, it was relatively weak. This could be explained by the fact that in the experiments of Aug. 22, 1957, the freshly separated mitochondria already had a lowered phosphorylating activity (P:O coefficient about 1) and relatively low optical density. In this experiment, when the solution which did not contain anserine was "aged" for 40 min, the process of phosphorylation practically stopped, though further decrease in optical density compared to the starting solution could not be great. The presence of anserine in the solution did not increase the intensity of phosphorylation or the optical density of the mitochondria. Anserine evidently only stabilizes the morphological structure of the mitochondria, delays the period of their progressive destruction, and therefore maintains their ability for respiratory phosphorylation. We should emphasize that this action of anserine is independent of its buffer properties.

5. According to the literature, "aging" of the mitochondria causes increase in the activity of their "latent" ATP-ase [15, 16] which controls the decrease in P:O coefficient because of the rapid splitting of the ATP which forms. In this connection we have carried out experiments to determine the intensity of splitting of ATP by the enzymes of the mitochondria, "aged" for equal periods in solutions I and IA. The ATP-ase activity was determined by the amount of mineral phosphate split from ATP under the influence of a 0.2 ml sample of mitochondria. The experimental samples with a volume of 2 ml were prepared as follows: to 1.8 ml of buffer solution (pH 7.4) which contained 100 μ moles of trihydroxymethylaminomethane (Tris), 10 μ moles of $MgSO_4$, and 10 μ moles of ATP, was added 0.2 ml of mitochondria sample. The temperature of the buffer solution and also the experimental sample during the 10 min incubation was 26°. Enzyme processes were stopped by addition of 2 ml of 5% trichloroacetic acid at the beginning of incubation and after 10 min. The results are given in Table 3.

According to the data of Table 3 the activity of ATP-ase in the mitochondria "aged" in solution IA for an hour has practically not increased and after 2 hours has increased only 10%. In the mitochondria "aged" in solution I, the ATP-ase activity increased somewhat more: after an hour, by 9%, and after 2 hours, by 20%. Hence, the presence of anserine inhibits somewhat the increase in ATP-ase activity during "aging" of the mitochondria. However, in the control sample the increase in ATP-ase activity was so slight that it could not explain the definite decrease in P:O coefficient after an hour's standing of the mitochondria at room temperature.

Thus, anserine keeps the mitochondria from separation of respiration and phosphorylation, but this cannot be explained by its buffering action and only in slight degree is there a relation to lessened increase in ATP-ase activity in its presence. The effect of anserine also does not depend directly on its participation in the phosphorylation process and its favorable action on the structure of the mitochondria its preservation during the "aging" process. This conclusion is clearly confirmed by the results of the next series of experiments.

6. Mitochondria suspensions after "aging" for 1 hour in solution I (control sample) and in solution IA (experimental sample) were centrifuged at 8000 g. The supernatant liquid was removed and each of the two portions of mitochondria were suspended in solution I with the same composition. After repeated centrifuging

and removal of the supernatant liquid, that is, after washing from the mitochondria the anserine which was present in solution IA, a sample was prepared from both portions of mitochondria in solution I in the same way as before, that is, so that in 1 ml of sample there was a weight of mitochondria equal to that separated from 1 g of tissue. The samples for incubation, with a volume of 2 ml, were made up as follows: 1.3 ml of phosphate buffer (solution II), 0.3 ml of hexokinase solution, and 0.4 ml of the mitochondria in solution I. In this case the incubated control and experimental samples had practically the same composition, since the anserine of solution IA had been removed by washing out in the centrifuge. The results are given in Table 4.

TABLE 3

ATP-ase Activity of Mitochondria "Aged" in Solution I (control sample) and in Solution IA (experimental sample)

Date of experiment	Time of "aging" the mitochondria in min	Amt. of mineral phosphate split from ATP during 10 min incubation, in μ moles	
		control sample	experimental sample
2.XII 1957	0	2,85	2,90
	20	2,88	2,93
	40	2,90	2,93
	60	3,07	2,93
2.XII 1957	0	2,30	2,30
	60	2,53	2,30
	120	2,76	2,53

The mitochondria sample "aged" in solution IA (that is, the experimental sample), even though the anserine had been removed by washing, maintained an appreciably higher P:O coefficient than did the mitochondria "aged" in solution I (control sample). Thus, the favorable action of anserine is related to maintaining the structure of the mitochondria when they are "aged" and this assures a more effective respiratory phosphorylation. We should add that the pH in the experimental and control samples after incubation was exactly the same.

TABLE 4

Oxidative Phosphorylation in Mitochondria "Aged" in Solutions I and IA, With Later Washing on the Centrifuge

See Table 1 for the symbols

Date of experiments	Control sample			Experimental sample		
	ΔO in μ atoms	ΔP in μ atoms	P:O	ΔO in μ atoms	ΔP in μ atoms	P:O
9.VII 1957	11,3	5,2	0,46	13,7	19,8	1,44
10.VII 1957	8,4	0,8	0,1	12,1	12,4	1,02

7. For solution of the question of the specificity of anserine action, we carried out a series of experiments entirely analogous to those described in section 2, with only this difference, that "aging" of the mitochondria was carried out not only in solutions I and IA, but also in solutions which contained instead of anserine, carnosine (IC), histidine (IH) and β-alanine (Iβ). The content of all these compounds in the solutions was equimolar (0.05 M) with equal osmotic concentrations (0.37 M) which were attained by corresponding decrease in the amount of KCl in the solution. The pH was determined with a glass electrode and in all cases was 7.4.

The results are given in Table 5.

It was shown that when the mitochondria were "aged" for 40 min at room temperature in the IC solution, which contained carnosine, the oxidative phosphorylation (P : O coefficient) of the mitochondria their relative volume, and optical density were stabilized in an analogous way to that of solution IA, but in weaker degree. The presence of histidine in solution IH had no stabilizing action, and the addition of β-alanine (solution Iβ) even worsened the phosphorylation, lowering the P : O coefficient and in a number of cases increasing the degree of swelling of the mitochondria. Thus, although the action of anserine cannot be considered specific, yet it has a greater stabilizing action on the structure of the mitochondria when they are "aged" than does carnosine. We must add that when freshly prepared mitochondria are placed in a solution which contains anserine or carnosine, during the first 30 min, and sometimes for an hour there is a small increase in optical density of the sample compared to the initial value determined immediately after preparation, that is, the dipeptides reduce the volume of the mitochondria which was increased in their separation.

TABLE 5

Oxidative Phosphorylation in Mitochondria and Their Relative Volume (by nematocrit) After Standing for 40 min at 19.5° (in solution I, IA, IC, IH, and Iβ

Date of experiment	Solution	Relative volume of mitochondria residue in %	ΔO in μ atoms	ΔP in μ atoms	P : O
19.X 1957	I	100	9,9	8,5	0,86
	IA	84	10,9	15,6	1,43
	IC	85	11,6	13,0	1,12
	IH	92,5	12,5	11,0	0,88
	IB	101	12,6	8,6	0,69

A more detailed study of the effects of dipeptides and amino acids on the structure of the mitochondria of skeletal muscle and the processes of oxidative phosphorylation which occur in it should be the subject of further investigation.

SUMMARY

The addition of anserine to a suspension of freshly separated mitochondria from pigeon pectoral muscle does not affect the intensity of the oxidative phosphorylation processes which occur in them.

The addition of anserine and carnosine to the medium in which "aging" of the mitochondria occurs prevents them from swelling and maintains the connection between respiration and phosphorylation. Histidine and β-alanine do not have this action.

LITERATURE CITED

[1] S. E. Severin and N. P. Meshkova, Doklady Akad. Nauk SSSR 74, 549 (1950).

[2] N. P. Meshkova and N. A. Malysheva, Doklady Akad. Nauk SSSR 81, 247 (1951).

[3] S. E. Severin and N. P. Meshkova, Doklady Akad. Nauk SSSR 84, 105 (1952).

[4] S. E. Severin and N. P. Meshkova, Doklady Akad. Nauk SSSR 92, 807 (1953).

[5] N. P. Meshkova and N. N. Zaitseva, Doklady Akad. Nauk SSSR 92, 999 (1953).

[6] S. E. Severin and N. P. Meshkova, Problems of Muscle Biochemistry, (Izd. AN SSSR, 1954), p. 193.*

[7] S. E. Severin, N. P. Meshkova, and N. I. Razumovskaia, Doklady Akad. Nauk SSSR 103, 87 (1955).

[8] J. B. Chappell and S. V. Perry, Nature 173, 1094 (1954).

[9] L. Berger, M. W. Slein, S. P. Colowick and C. F. Cori, J. Gen. Physiol. 29, 379 (1946).

* In Russian.

[10] J. B. Sumner, Science 100, 413 (1944).

[11] K. W. Cleland, Nature 170, 497 (1952).

[12] R. E. Beyer, L. Ernster, M. Low, and J. Beyer, Exptl. Cell. Res. 8, 586 (1955).

[13] J. W. Harman and M. Feigelson, Exptl. Cell. Res. 3, 509 (1952).

[14] J. W. Harman and A. Kitiyakara, Exptl. Cell. Res. 8, 411 (1955).

[15] W. W. Kielley and R. K. Kielley, J. Biol. Chem. 191, 485 (1951).

[16] J. B. Chappel and S. V. Perry, Biochem. J. 55, 586 (1953).

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FURTHER IMPROVEMENT OF AN APPARATUS FOR THE ELECTROPHORESIS OF PROTEINS

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The construction of an apparatus for the electrophoresis of proteins with optical recording of the moving interface was described earlier [1, 2]. In the present paper we describe further changes in this apparatus which make it suitable for optical microelectrophoresis. We have also introduced some supplementary improvements which increase the accuracy of the work and greatly simplify the building of the apparatus.

In the types of microapparatus which now exist for optical electrophoresis [3-13], a glass cuvette is always used; its volume is one fifth to one-tenth that of the macromodels. The smaller volume of the cuvette is obtained either by decreasing the height (86 mm to 30 mm) or the thickness of the layer of protein solution (from 25 mm to 5 mm). As the literature indicates [14, 15, 22] the gap in the glass cuvette cannot be less than 2 mm. This is because the surface of the glass which contains the ionogenic group carries a large charge and so in a glass cuvette with a small opening there is strong endosmosis (electroosmosis), and the endosmosis overcomes the electrophoretic curve.

The impossibility of decreasing the opening of the glass cuvette reduces the possibility of using frontal electrophoresis in the microelectrophoretic apparatus and makes it important to use a more sensitive optical device, since the ordinary optical method based on the shadow principle of Foucault-Tepler is not sensitive enough for work with a microcuvette. Starting from this, some authors have used more sensitive devices based on the interferometer principle [16, 17, 18]. However, the use of an interferometer in electrophoresis is not convenient, since the interferometric picture is obtained on a screen and must be measured, treated mathematically, and a graph must be constructed from the resulting points (the electrophoretic curve). In addition, using the interferometric method of measurement, it is impossible to obtain an exact boundary for the refractive index gradients and so the accuracy of the method [13] is somewhat lessened.

From all this it follows that the cuvette for electrophoresis should be constructed of a material which in contact with water has a small ζ-potential and permits decreasing the opening of the cuvette without danger of endosmosis. Plexiglas is such a material. This was shown by our experiments which indicated that if glass in the cuvette was replaced by plexiglas, the effects of endosmosis were sharply reduced and the opening of the cuvette could be made 0.4-0.8 mm, that is, a three- to five-fold reduction. Thus, it is possible to construct a microcuvette with the same volume that is used in microelectrophoresis [4, 7], but with a thicker layer of protein (25 mm). Since the sensitivity in recording the refraction gradient in our cuvette is the same as in the macrocuvettes, we can use the ordinary optical recording device. This permits the use of such a microcuvette in the ordinary electrophoresis apparatus.

We successively built and studied plexiglas cuvettes with cross sections in the U-tubes of 3 x 25 mm, 1.5 x 25 mm, 0.8 x 25 mm, 0.4 x 25 mm, and 0.2 x 25 mm.

The most suitable cuvettes for microelectrophoresis were those with openings of 0.4 mm and total volume 0.42 cc, and 0.8 mm with volume 1 cc. In running each experiment with these cuvettes it was necessary to have a total of 0.2-0.5 ml of serum or 10-20 mg of protein.

Construction of Microcuvettes from Plexiglas

The cuvettes were constructed entirely of plexiglas. They had their own electrophoretic chamber (optical element), consisting of a U-tube with rectangular section (0.4 x 25 or 0.8 x 25) and two electrode vessels. The electrode vessels could be constructed in one of the two variants of Fig. 4 (Diagrams 29 or 49), which did not differ from each other in principle. The basic work was carried out with the first variant (Diagram 29).

Electrophoretic Chamber – Optical Element

We worked out a method of constructing the optical element which was simpler than that previously described. The cuvette could be built in the laboratory without using any complex instruments. The construction of the optical element of the cuvette was the most difficult, and so we will give the most detailed attention to this.

Construction Details of the Optical Element

In Fig. 1, diagrams 1-8, we give the form and dimensions for the construction of the U-shaped vessel, and in diagrams 9-14 we give the successive steps in its assembly and fastening together.

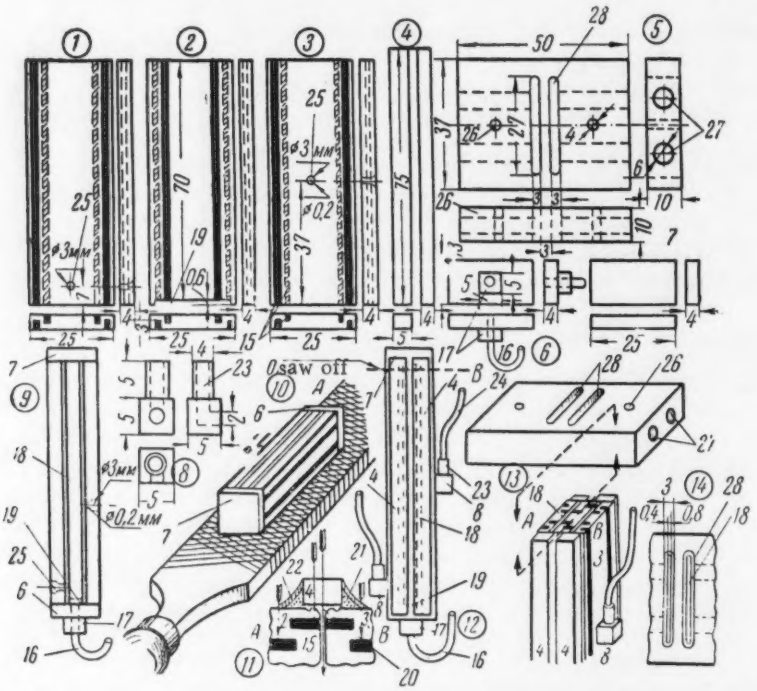

Fig. 1. Details of the optical element and the successive stages in assembling it. Diagrams 1, 2, 3) Side walls of the U-shaped vessel; 4) front and back walls of the vessel, showing its optical window; 5) cross section of the plate which connects the U-tube with the electrode vessel; 6, 7) bottom and top of the optical element; 8) sleeve passing capillary (25); 9) side walls with attached bottom and top; 10) method which permits exact polishing of the surfaces of the side walls; 11) part of the side walls (2, 3) with attached plexiglas window. (The light in the cuvette passes only through the protein solution, but not through the side walls of the cuvette); 12) the same as in diagram 9 but with attached window and sleeve for passage of capillary; 13) moment of fastening together the cross piece on the surface of the optical element, formed after cutting at A-B; 14) appearance of the optical element from above. Shows the coinciding of the opening of the U-shaped vessel (18) and the slit of the cross piece (28).

From 4 mm plexiglas we cut out three plates (Fig. 1, diagrams 1, 2, 3) with dimensions 25 x 70 mm. These plates, the side walls of the U-tubes, must be made opaque to light passing through them (course of the light in the cuvette). Then the light from the light source will pass only through the solution being studied (Diagram 11). The metallic diaphragm with the narrow slit used in the macroapparatus is not successful in the microcuvette, since it lessens the amount of light passing through, and without this it is difficult to match exactly the narrow slit of the diaphragm with the opening of the cuvette. In order to prevent the walls of the cuvette from passing light we found it sufficient to cut two grooves to a depth of more than half the thickness of the wall (about 3 mm deep) and fill them with an opaque, nonconducting mass (15). Such a mass could be prepared from powdered plexiglas placed in dichloroethane with the addition of a black lacquer (Fig. 1, diagrams 1, 2, 3, and 11, explained above). The drawings of the side walls of the cuvettes are given in three projections.

In one of the strips a groove is deepened with a file to a depth of about 0.5 (or 0.9 mm) reaching to 1 mm from both sides (2). In the other two plates (1 and 3) hollows are bored out with a diameter of 3 mm and a depth of 3.0-3.5 mm. The rest of the cuvette walls are bored with a 0.2 mm drill or the end of a fine needle in such a way that the end hardly passes over the surface of the plexiglas. An opening is drilled in the first plate at a distance of 7 mm from the lower edge, and in the third at 37 mm. Through these capillary openings (25) at the time of layering part of the protein is removed by a vinyl chloride capillary from below (16). At this point a clear interface of buffer protein is recognized.

Two plates with diameter 25 x 13 mm are cut from 4 mm plexiglas for the bottom and top of the electrophoretic chamber (Diagrams 6, 7). The top cover (7) plays a subsidiary role. It serves to fix in a definite position the side walls which are fitted and polished to hold the optical plexiglas window (4). In the center of the lower plate (6) is bored an opening for the capillary which introduces the solution for layering. A vinyl chloride capillary can be made from ordinary wire with a vinyl chloride insulation by pulling out the copper wire. The diameter of the copper wire should not be more than 0.2-0.3 mm. The volume of the capillary with a length of about 100 mm is 0.003-0.007 cc. The vinyl chloride capillary (16) is forced through the opening in the bottom with a drop of chloroform. For greater strength a 5 x 5 mm block is fastened below (17) the opening in the center of (6). If the side walls are to be attached strictly parallel and with the same height for the opening, it is necessary to use a metallic pattern 0.4 or 0.8 mm thick. The opening and volume of the U-tube in the electrophoretic vessel will depend on the thickness of the pattern. It is possible to use a steel millimeter ruler of the proper thickness, cutting two pieces from it 65 mm long.

The side plates and the pattern are put together in the following order: plate (1), metallic pattern, plate (2), metallic pattern, plate (3). The capillary opening must be turned in to the slit, but the wide opening must be outward. The plates and metallic patterns are pressed together with a clamp or vice, and are matched at the upper and lower edges so that they form an even plate held together at the top by the cover (7) and at the bottom by the plate with the capillary (6), and several drops of chloroform are applied to the plates which are touching. When the glued places have dried well, the clamp is removed and the metallic patterns are pushed out. In their place, between the side walls there remains a narrow canal slit (18) 70 mm long and 0.4 (or 0.8 mm) wide and 25 mm deep (Diagram 9). The two canals are joined by a groove (19) in the middle partition (Diagram 2), and here the open end of the capillary for layering is brought in (16).

The optical element is polished as shown in diagram 10 until the planes are polished to mirror glass. The other edges are similarly polished. During this time the parallel edges are continually examined with calipers. When the polishing is finished, the tip of a lancet is inserted into the groove and removes a small edge with dimensions 0.3 x 0.3 mm (20) along the whole length of both sides of all the grooves (Diagram 11). This edge is needed so that at the time of gluing a drop of chloroform will not leak onto that part of the optical plexiglas (4) which serves as a window for the light (Diagrams 11, 12; the dotted lines correspond to the opening in the cuvette, 13). Four plates with dimensions 5 x 75 mm are cut from the "optical" plexiglas (diagram 4). They serve as windows for the optical element and appear as front and back walls of the U-shaped electrophoretic vessel. When the windows (4) have been sealed on, every joint is sprinkled with finely ground plexiglas powder, evenly spread along the joint. Drops of chloroform are dripped onto the powder from a fine pipet, passing along all the joints successively (21). After a day, all the joints are covered with "plexiglas lacquer" (22), prepared by dissolving plexiglas filings in dichloroethane to a thick sirup. The lacquer fills all the hollows and cracks between the particles of powder and somewhat increases the period of service of the cuvette and improves its insulating properties. The joints so prepared are shown in cross section in diagram 11.

Two sleeves are prepared from 5-6 mm of plexiglas with the dimensions shown in diagram 8. Into the opening of the cylindrical part (23) is tightly inserted a vinyl chloride tube (24) with inner diameter 0.8-1.5 mm, obtained from wire of this diameter by the process described above. Both sleeves are glued into place for the capillary outlets (25), diagram 12.

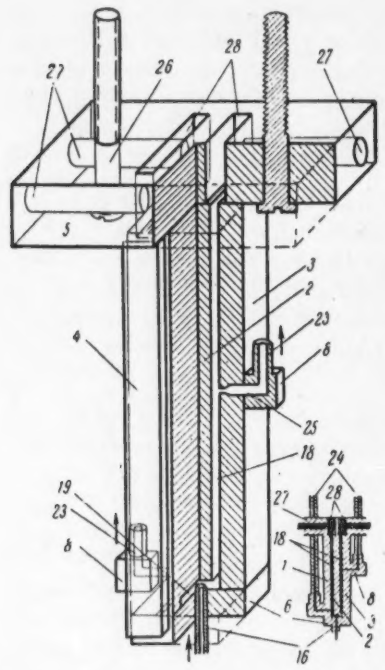

Fig. 2. Optical element of the U-shaped vessel.
Part of the front and side walls are removed and the places cut out are cross hatched. This permits a clear view of the course of the canals of the U-shaped vessel (18, 19), the layering (16), and exhaust (25) capillaries. In the lower right corner is given the plan of the same optical element. The plexiglas walls are cross hatched.

The cross piece (diagram 5) is a connecting link between the electrode vessel and the U-shaped vessel of the optical element. It is cut from 10 mm plexiglas with dimensions 37 x 50 mm. In it there are the opening (26), the canals (27), and the slits (28), just as shown in diagram 5.

When the optical element is sufficiently dry its upper part is accurately sawed off along the line A-B (diagram 12) so that there is an even plane perpendicular to the electrophoretic canals (18). This plane of the optical element is glued to the cross piece (5), diagram 13. In diagram 14 it is shown that the openings of the canals (18) of the U-shaped vessel coincide with the slots (28) in the cross piece. Powdered plexiglas with chloroform and "lacquer" hermetically seals all the joints, especially in the slot (28) so that there is no capillary connection between the electrophoretic canals by which a "leakage current" could pass. The general form of the complete optical element with cross piece is shown in Fig. 2. In order to show the various details and the course of the electrophoretic canals, part of the front and side walls are shown removed (the place of the supposed cutting away is cross hatched). The notation is the same as in Figs. 1, 3, and 4.

Electrode vessel. To prevent denaturation of the protein or coagulation of the colloids, which might occur from secondary reaction on the electrodes, the electrodes are removed as far as possible from the protein by placing them in a large volume of buffer. A very large vessel cannot be used in a microapparatus since the current strength does not surpass 1-3 mA. A volume of 100-150 ml of pure buffer is sufficient. Also, after each experiment the cuvette is washed with 20-30 ml of pure buffer and the buffer itself is constantly renewed. We ran 15-18 experiments without overloading.

The electrode vessel is also made of plexiglas. Its dimensions and form are shown in Fig. 3, diagram 30 and in Fig. 4, diagram 49, which shows the general form of the complete cuvette with the electrode vessel. Besides the cuvette with silver chloride, nonpolarizing electrodes which we described earlier [2], we successfully used a carbon electrode with an agar bridge. It is prepared as follows: two Schott funnels (31) filled with 3% agar in a saturated KCl solution are used instead of the silver chloride electrode; the upper part of the funnel is filled with saturated KCl solution (32) in which is placed a graphite electrode (33). Best results are given by a graphite rod for spectrum analysis.*

The vinyl chloride tubes of the electrode vessel and the exhaust capillary (24) are connected to glass stopcocks (34). In order to join the vinyl chloride tube with the stopcock, one of the ends (35) of the stopcock is fused in a flame until the diameter of the opening becomes such that the end of the vinyl chloride tube fits into it hermetically. All the stopcocks are mounted on a plexiglas panel (36), Fig. 3, with dimensions 140 x 100 mm.

* The use of a Schott funnel permitted assurance of a hermetic seal to the cuvettes, since the porous filter plate filled with agar gave a hermetic seal which kept its conductivity.

Assembling the cuvette. The optical element is set between the electrode vessels in such a way that the canal openings (27) of the cross piece (5) coincide with the openings in the side walls of the electrode vessels. At the places of contact several drops of chloroform are introduced. The back walls (36) are attached simultaneously. When they have dried, all the joints are sealed with powder and plexiglas lacquer (diagram 30, Fig. 3).

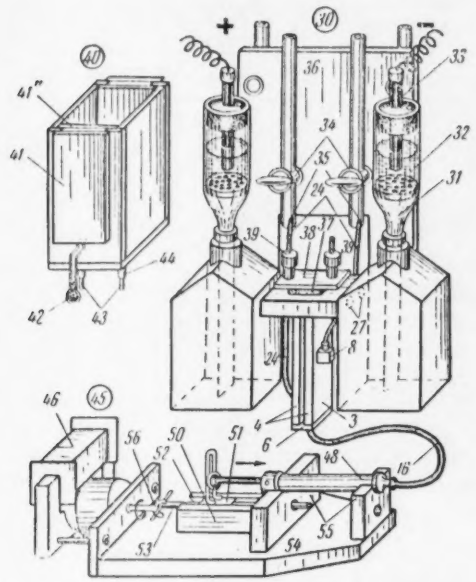

Fig. 3. General view of the enclosed cuvette with electrodes and layering device. Diagram 30) Cuvette with graphite electrodes (33) and agar bridges (31, 32); 45) layering device; 40) general view of thermostat.

In order to seal shut the outlet from the canals of the U-shaped vessel, we use a plexiglas plate (38), a piece of soft plastic (37) and two supports (39).

Loading the cuvette. When the microcuvette is finally dry it is tested for hermetic sealing and leakage currents. When no leaks are found, the cuvette is washed with a strong stream of water and filled for one or two hours with 20-30% alkali solution. Then it is carefully washed with running water. The plexiglas window (4) is washed on the outside with benzine on cotton and carefully wiped dry. The cuvette is filled with buffer. The plastic pad (37) covers the outlet of the electrophoretic canals (28).

A 16 mm cork is placed in the opening for the electrodes; it has a glass tube with a bulb at the end in it. With pressure from the bulb, the buffer is driven over from one electrode vessel to the other while the stream of liquid pushes out air bubbles from the narrow capillary spaces of the U-shaped central element. When the plastic pad is raised slightly, the accumulated air passes out and the pad is fastened tightly. Both electrode vessels are filled full with buffer so that no air remains anywhere. The electrodes are inserted tightly in the electrode openings. The cuvette is ready for work.

Construction of the Thermostat

When a current is passed, the protein and buffer solutions are heated. The cuvette is cooled to prevent disturbance of the interfaces by the convection currents. Since heat is evolved especially where the current density is highest, that is, in the narrow canals of the U-shaped vessel, only the optical element of the apparatus is cooled by placing it in a thermostat. The electrode vessel remains outside. This permits the use of a thermostat with a very small volume. Such a thermostat is easily cooled to a low enough temperature even with a domestic refrigerator, which is also used to keep the proteins being studied [19].

The general form of the thermostat is shown in Fig. 3, diagram 40, from which its construction is clear. The thermostat window (41) is made of "optical" plexiglas. To prevent the window from dimming, it is made double (41 and 41) and the moisture in the space between the plates is absorbed by a calcium chloride tube (42). The water in the thermostat is cooled with a coil (43) in which antifreeze is circulated: a mixture of water and alcohol (1 : 1). At the end of the copper coil is placed a plastic coupling (44), by which the tube passing through the bottom of the thermostat is kept sealed in.

Layering Apparatus

Principle. The resolving power of the electrophoretic apparatus depends greatly on the quality of the original interface between the protein solution and the buffer side liquid. The initial peak in the electrophoretic curve should be as sharp as possible, approaching to a single line. In this case the refraction gradient is shifted sharply. Evidently, the more the initial interface is weakened, the greater will be the space needed to separate the fractions which are formed so that their separation can be recorded. In the microapparatus this would be impossible, since the space for the separation of the fastest fraction does not exceed 30 mm. In the case of

multicomponent systems such as blood serum or plasma an unclear interface caused by poor layering can lead to poor quality in the optical recording system [12].

The hydrodynamic method described by Svensson has a series of advantages over the other methods of producing layering, but from the point of view of the microapparatus it is unsuitable for the following reasons.

1. A large amount of the substance under study is not used and remains in the layering capillary and pipette.

2. The rate of layering differs at the beginning and end and depends on the height of the column of liquid in the layering pipette.

3. Regulation of the rate of addition through the stopcock is difficult. The shock at the time of layering injures the interface.

4. In a wide layering capillary there is mixing of protein and buffer. To avoid this, the following improvements were introduced into the system for producing the interface:

1) the cross section of the layering capillary was reduced 29-fold, the volume of "dead space" (unused portion of the protein) was thus reduced from 0.5 ml to 0.007 ml, that is, 72 times; this prevented mixing of the solutions in the capillary;

2) layering through such a narrow capillary must be produced by an even, positive pressure. This pressure is furnished by a syringe whose piston is moved by a special mechanism set in motion by a synchronous motor with a reducer. As a result of the even layering, as good an interface is obtained as with a Tiselius apparatus, but better because it permits running a large number of experiments without reloading the cuvette (20). Also, the Tiselius cuvette cannot be built in the laboratory, but the cuvette with the hydrodynamic layering device can be built comparatively easily.

Construction of layering device. The device consists of the following parts (Fig. 3, diagram 45): Warren type synchronous motor (46) with reducer; "Record" syringe, 1 ml, (48), with a thin point whose end is inserted in the vinyl chloride capillary (16) which leads to the central optical element. The structure (50) converts the even, rotatory motion of the motor into the direct progression of the carriage (51), that is, the motion which operates the piston of the syringe. This structure consists of the carriage (51) which moves in a groove (52), and a micrometer screw (53) which moves it along the groove. For building this apparatus we used the microscrew from an old school microscope. All the parts were mounted on a general base (54). The syringe (48) was supported on a plexiglas stand (55). After layering, the whole system was returned to the original position. For this we used a cardan joint (56) and a key (not shown in the diagram) by which the micrometer screw was turned by hand in the reverse direction to return the carriage (51) to the starting position. The general view of the layering apparatus is shown in Fig. 3, diagram 45 where the interrelations and connections of all the parts of the mechanism are shown.

The Use of the Coulometer in Electrophoresis

As a source of constant current, a rectifier with electronic voltage stabilizer is most often used. In the case of the use of a coulometer in electrophoresis such strict stabilization is not necessary and it is quite enough to use a ferroresonance stabilizer. To measure the amount of electricity used in the experiment, it is not necessary to measure the current strength and the length of the experiment, but only to construct an automatic integrator of current strength with time, that is, a coulometer. The advantage of using a coulometer for electrophoresis has been considered by one of us in paper [21] and is clear from the following considerations: accuracy of determining mobility depends chiefly on stability of thermostatic control and regime of feeding the solution, but even when these conditions are satisfactory the passage of the electric current through the solution produces heat in it, mutual diffusion of the solutions with different specific resistances, polarization and other processes which change the resistance of the cuvette and so change the current strength. Measurement of the mobility with the aid of a coulometer removes these difficulties. Although the current strength constantly changes, the resistance of the cuvette is variable and the mobility of the colloid particles is also uneven, yet the results of the transfer of the protein fractions during the whole experiment is proportional to the amount of electricity, Q, used in the experiment. This permits us to reduce the requirements to thermostatic control and current stabilization.

Mechanical coulometer. In principle our mechanical coulometer is a direct current commutator motor connected to a rotary counter (58). A definite number of rotations of the armature gives the number of coulombs entering the experiment.

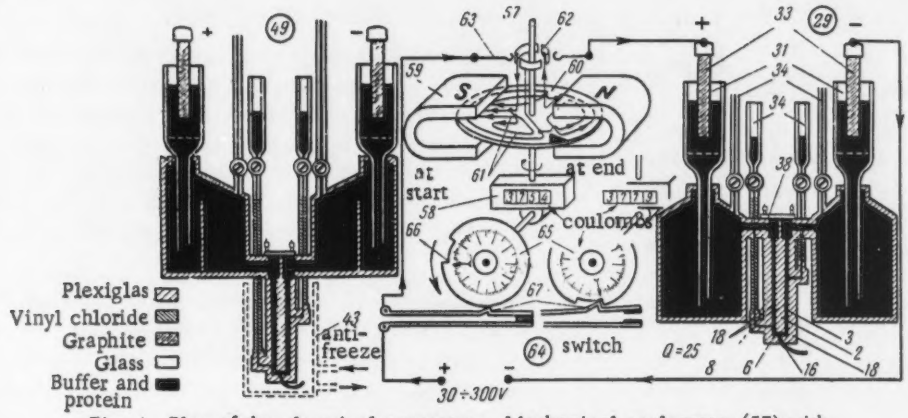

Fig. 4. Plan of the electrical apparatus. Mechanical coulometer (57) with automatic device for starting the experiment (64) after passage of a previously determined number of coulombs of electricity. At the sides are shown the possible variants of the electrode vessels (29, 49).

The coulometer is best operated on a separate block in series with the cuvettes of the electrophoretic apparatus (Fig. 4, diagram 57). Between the poles of the two permanent magnets (59), the aluminum rotor disc (60) turns in a horizontal plane, with three coils (61) at angles of 120° on it. The coils are wound on a special form with PE wire, 0.07 mm, 1750 turns. The beginning of one and the end of the next coil are joined to each other, as shown diagram 57, and lead to the commutator plates (62). Current is supplied to the rotor through miniature brushes (63). When current is passed, the armature rotor begins to turn, and the rate of turning depends on current strength in strict linear relation. The mechanical coulometer is calibrated with a copper coulometer [23].

The mobility is calculated by the formula

$$U = \frac{S \cdot q \cdot \mu}{Q \cdot g} = cM^2U^{-1}\text{sec}^{-1},$$

where U is the mobility, S is the path followed by the fractions in cm, q is the cross section of the plane in cm^2, μ is the specific conductivity in reciprocal ohms, Q is the amount of electricity in coulombs, g is the magnification factor of the electrophoretic apparatus.

Automatic performance of the experiments. The use of the mechanical coulometer permits automatic electrophoresis. In Fig. 4, diagram 64, there is shown one of the simplest variants [21] of the construction, which consists of a disc (65) which turns with the coulometer armature and completes one full revolution with 100 revolutions of the armature. There are 100 divisions on the disc. Each division corresponds to one coulomb. At the zero mark on the disc a groove (66) is made against which at a definite position there juts out a spring contact (67). The disc is connected by hand to a panel (not shown in the diagram) which can be set at any desired number of revolutions of the armature, that is, coulombs (diagram 64, on the left, 25). During the experiment, the disc (65) is slowly turned in the direction indicated by the arrow to the starting zero position. The projecting spring contact (67) enters the groove (66) (diagram 64, on the right) and contact is broken. The experiment is switched off and the coulometer stops.

Results of Testing the Apparatus

In Fig. 5 we give the electrophoretic curves for blood serum of rabbits and bulls, studied at the same time in macro- and microapparatus, with cuvettes which have different openings in the electrophoretic canals – 0.8, 0.4, and 0.2 mm.

When curves I, II, and III are compared, it is quite obvious that curves I and II agree completely, and I and III are close to each other. Curve VI shows that even with such small openings in the cuvette as 0.2 mm it is still possible to separate the serum into fractions by electrophoresis. However, the manufacture of cuvettes with such narrow openings is technically difficult. All the studies were carried out with serum which contained 2.5% protein.

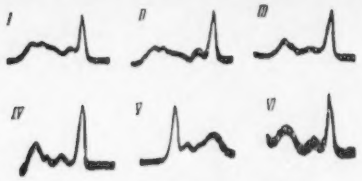

Fig. 5. Photograph of the electrophoretic curves of serum, studied in the macro- and microapparatus. I, II, and III) Curves of the same serum studied in cuvettes with different openings in the U-shaped vessels: I) in a macrocuvette (3-5 ml of serum for an experiment); II) in a microcuvette with 0.8 mm opening (0.5 ml of serum); III) in a microcuvette with 0.4 mm opening (0.25 ml of serum); IV and V) ascending and descending curves (microcuvette with 0.8 mm opening); VI) ascending curve with a microcuvette with a 0.2 mm opening (0.3 ml of serum in one experiment).

As the data show (Table) the results with the same serum in the macro- and microapparatus with cuvettes of different openings in the U-shaped vessels show sufficiently comparable results. However, the micromethod is indispensible in work with small laboratory animals, especially when blood is taken repeatedly. The micromethod can be widely used also in the clinic, since it is possible to carry out optical electrophoresis on seriously ill patients or young children.

In comparison with paper chromatography, optical electrophoresis shows great accuracy and the absence of artifacts compared to the adsorption on filter paper ("albumin strips," adsorption of lipoproteins, etc.). In spite of the clear advantage of frontal electrophoresis over paper chromatography, its wide introduction has been hindered by complexity and cost of apparatus for optical electrophoresis, and the large amount of serum needed for each study. This work largely overcomes these objections to optical electrophoresis with a free interface.

TABLE

Comparative Percent Content of Protein Fractions in the Same Serum and Their Mobility in the Macro- and Microapparatus

Type of electrophoretic apparatus	Content of different proteins, in % of total amount				Mobility of different fractions, in 10^{-5} cm^2 v^{-1} sec^{-1}			
	albumin	globulins			albumin	globulins		
		α	β	γ		α	β	γ
Macroapparatus	39.7	9.2	8.1	43.0	7.5	5.7	3.8	1.5
Microapparatus	38.4	8.1	9.5	44.0	7.7	6.0	3.9	1.4

SUMMARY

As a result of these experiments we have shown that if the glass walls of the U-shaped vessel of the electrophoretic cuvette are replaced by plexiglas, endosmosis, which usually interferes with electrophoresis, is greatly lessened, and the openings of the canals which in glass cuvettes are not less than 2 mm can be decreased to 0.8 and even 0.4 mm. This permits a decrease in the volume of solution needed for the experiment to one fifth, while retaining the same sensitivity as in the standard experiment. Thus, it becomes possible to use the ordinary apparatus with the Foucault-Tepler optical device, and to shift from the macromethod (2 to 5 ml of serum per experiment) to the micromethod (0.3 to 0.5 ml of serum) only by a change in the cuvette.

We have described the construction of the microcuvette, entirely made of plexiglas, and have worked out the technology of its manufacture by the staff of the biochemical laboratory. Particular attention has been paid to the optical element of the U-shaped canals, which differ from the earlier description [1, 2].

Since in the microcuvette the original buffer-protein interface should be very sharp, we have constructed a special layering device which consists of a syringe whose piston is slowly and evenly operated by a synchronous motor.

In measuring the mobility, we found it more accurate not to measure the current strength and duration of the experiment, but to use an automatic integrator of the current strength with time, that is, a coulometer. We have described a mechanical coulometer, simple in construction, which works well at a current strength of 0.2 mA and above. We have given a plan which permits automatic operation of the electrophoresis experiment with a given amount of electricity, and the formula for calculating the mobility of the protein fractions using measurement by the coulometer.

Parallel studies of the same serum in the macro- and microcuvettes (0.8 and 0.4 mm) have showed sufficient reproducibility in the relative percent of the protein fractions and the electrophoretic mobility.

LITERATURE CITED

[1] G. V. Troitskii, Biokhimiia 16, 592 (1951).

[2] G. V. Troitskii and I. I. Radionov, Biokhimiia 20, 431 (1955).

[3] H. Antweiler, Kolloid. Z. 115, 130 (1949).

[4] H. Antweiler, Mikrochem. 37, 561 (1951).

[5] H. Antweiler, Chem. Ing. Technik. 24, 284 (1952).

[6] W. Geissen, B. Schuler, and H. Sehuster, Klin. Wochenschr. 28, 751 (1950).

[7] H. Labhart and H. Staub. Halv. chim. acta 30, 1954 (1947).

[8] W. Lotmar, Helv. chim. acta 32, 1847 (1949).

[9] W. Lotmar, Rev. Sci. Instrum. 22, 886 (1951).

[10] F. Struss and G. Urban, Wiss. Z. Univ. Halle, Math. Nat. 4, 359 (1955).

[11] H. Antweiler, Die quantitative Elektrophorese in der Medizin (Göttingen und Heidelberg, Berlin, 1952), p. I.

[12] L. Longsworth, Analyt. Chemistry 25, 1074 (1953).

[13] E. Wiedemann, Handbuch der physiologisch. pathologisch. chem. Analyse, p. 54 (Göttingen und Heidelberg, Berlin, 1953).

[14] H. Abramson, M. Moyer, and M. Gorin, Electrophoresis of Proteins and the Chemistry of Cell Surfaces, (Reinhold, New York, 1942).

[15] H. Svensson, Electrophoresis by the Moving Boundary Method, Uppsala (1946).

[16] H. Svensson, Transactions of Instruments and Measurements Conference, p. 58 (Stockholm, 1952).

[17] G. Kortüm, Handbuch der physiologisch, pathologisch. chem. Analyse, p. 441 (Göttingen und Heidelburg, Berlin, 1953).

[18] A. N. Zakhar'evskii, Interferometers (1952). *

[19] I. I. Radionov and G. V. Troitskii, Labor delo, 3, 48 (1957).

[20] G. V. Kobozev, Scientific Session, Crimean Med. Inst. (Simferopol, 1956). p. 18.*

[21] G. V. Kobozev, Uk. Biokhim. Zh. 29, 375 (1957).

[22] M. Bender, J. Colloid Sci. 9, 400 (1954).

[23] D. MacInnes, The Principles of Electrochemistry (Reinhold, New York, 1939), p. 29.

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* In Russian.

THE POLYGLUCOSIDES OBTAINED AS A RESULT OF THE DIGESTION OF LIVER GLYCOGEN BY α-AMYLASE

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There have been indications in the recent works of a number of authors that the structure and properties of glycogen-type polyglucosides were subject to considerable variation, consisting in differences in degree of branching, length of terminal chains, iodine staining, molecular weight (M), degree of hydrolysis of enzymes, ability to form complexes with proteins and the content of various fractions which may be resolved by adsorption analysis. It was also shown that changes in the structure and properties of glycogens took place during a number of pathological conditions, notably during diabetes, von Gierke's syndrome and many other diseases.

However, the present information on the subject has not yet exhausted the multitude of forms of glucose polymers found in animal organisms; at the same time, the establishment of the existence of a number of differences in structure and properties of various glycogens fails to explain a number of new facts which have recently come to light.

Thus, it was shown in 1955, that animal tissues contained the enzyme, α-1,6-dextranglucosidase, capable of splitting α-1,6-bonds in glycogen molecules up to the branching points, with the formation of glucose [1-3]. α-1,6-Dextranglucosidase differed from other enzymes which, though capable of splitting α-1,6-bonds of glycogens (at the "branching points") were unable to split dextrans; the dextranglucosidase must therefore be considered to have an independent function in the body. Similar enzymes were previously known only in bacteria and molds. The presence of dextranglucosidases in animal tissues may indicate the presence there of dextran-like polyglucosides.

Wolfrom and Thompson [4] in 1956 found among the products of incomplete acid hydrolysis of liver glycogen a trisaccharide which they identified as isomaltotriose, that is, a trisaccharide with two α-1,6-glucosidic bonds. This trisaccharide is formed during the incomplete acid hydrolysis of dextran, while the acid hydrolysis of glycogen generally yields maltotriose, a trisaccharide with two α-1,4-bonds, and panose, which contains one α-1,4 and one α-1,6-bond. The observation of Wolfrom and Thompson [4] indicates the need of a revision of the existing concept of glycogen structure, since the glycogen molecules contain α-1,6-bonds not only in the branching points, but also in the chains (both internal and terminal). Another explanation would involve the assumption that liver glycogen contains an admixture of another polyglucoside, whose structure is similar to that of dextran. There is thus a possibility that liver (and possibly also other tissues) contain polyglucosides which differ from glycogen.

The search for a suitable α-1,6-dextranglucosidase substrate in animals revealed the fact that amylolysis of liver glycogen with salivary amylase yielded a number of polyglucosides which differed from glycogen in that they did not stain with iodine and that their molecules contained a considerable proportion of α-1,6-bonds [5].*

* In the future these compounds will be referred to simply as polyglucosides, in distinction from glycogen and dextrans. The present communication deals with the structure and properties of these polyglucosides.

EXPERIMENTAL

Preparation of Polyglucosides

Earlier preparations employed the "alkaline" or the "aqueous" methods described before [5]. In later experiments the methods employed were as follows.

The livers of 1 or 2 rabbits were homogenized with an equal volume, by weight, of 5% trichloroacetic acid. The suspension was allowed to stand for one hour and was centrifuged; the extract was dialyzed 24 hours in order to remove the trichloroacetic acid, the pH of the solution was adjusted to 6.8-7, 5-10 ml of filtered saliva was added and the mixture incubated at 37°. If the mixture showed a negative reaction to iodine (absence of color) after two hours, thus, indicating that the enzyme was active, it was left in the bath for 24 hours, after which another quantity of saliva was added and the incubation continued for another 24 hours. The enzyme was then inactivated with trichloroacetic acid and the extract was again dialyzed against tap water, followed by dialysis against distilled water, in order to remove the products of glucose amylolysis; the dialisate was then treated with five volumes of ethanol. A slight precipitate, usually adhering to the glass of the vessel, appeared at this stage. This precipitate was thrice reprecipitated from an aqueous solution by addition of alcohol and finally dried in vacuo over calcium chloride. Up to 500 mg of the precipitate was obtained from one batch, but in some experiments the yield was considerably lower. The precipitate dissolved easily in water and produced a bright blue color with the anthrone reagent. The moisture content of the preparations varied between 10 and 15%.

It will be seen from Table 1 that the preparations contained practically no nitrogen, while their specific rotation in aqueous solution fell within the limits of that of dextrans and glycogens. Acid hydrolysis of the product yielded 85-90% of glucose.

TABLE 1
Comparative Data on the Properties of Glycogens, Dextrans, and Polyglucosides

Preparation	Nitrogen,%	Moisture, %	Sugar, % of theory	$[\alpha]_D^{20}$	Color with iodine
Glycogen	0,03—0,1	5—10	90—95	192°	+
Dextran	0	15—18	82—85	196°—200°	—
Polyglucoside	0,00—0,05	10—15	85—90	195°—200°	—

Glucose was estimated chromatographically and by fermentation with specific yeasts [5]. The fermentation of hydrolysates was determined chromatographically and by the disappearance of reducing power. It was established by both methods that the only monosaccharade formed after acid hydrolysis was glucose and that therefore the preparation was a polyglucoside. Investigation of the products of incomplete acid hydrolysis of the preparation by means of descending chromatography revealed the presence of isomaltose predominantly to maltose. When the three polysaccharides, namely, glycogen, dextran and the polyglucoside were hydrolyzed under the same conditions, that is for 30 min, 1 hour or 1.5 hours in 0.5 N HCl, the following results were obtained (Fig. 1). Isomaltose only could be detected in hydrolysates of dextran intradex; chromatography of glycogen hydrolysates showed the presence of a small spot corresponding to isomaltose and a large intense spot corresponding to maltose; the reverse ratio held for the polyglucoside hydrolysate, which in chromatographs showed intense isomaltose spots, similar to the maltose spots obtained with glycogen hydrolysates, and small faint maltose spots. The chromatographic solvent employed in the present work was the mixture butanol-acetic acid-water (4:1:5). The spots were detected with aniline hydrophthalate.

As shown by I. S. Lukomskaia [6], isomaltose spots differ from those of maltose not only by their R_f values, but also by their coloration after treatment with aniline hydrophthalate. Schwimmer [7] showed earlier that oligosaccharides composed of glucose units joined in α-1,6-bonds differed from those containing the glucose units joined in α-1,4-bonds in the color they produced on chromatograms sprayed with a mixture of aniline, diphenylamine and phosphoric acid. The difference in color depends on the availability of the 4 free OH groups of the glucose moieties for the reaction.

It was found in the present experiments (see below) that different colors were given by dextrans and glycogens on electropherograms, after periodate oxidation and subsequent treatment with Schiff's reagent.

Fig. 1. Chromatographic pattern of the products of partial hydrolysis of dextran, glycogen, and polyglucosides. 1) Dextran; 2) polyglucoside; 3) glycogen; a) isomaltose; b) maltose; c) glucose.

The chromatographic demonstration of the predominance of isomaltose over maltose in acid hydrolysates of polyglucosides suggested that these compounds contained a large proportion of α-1,6-glucosidic linkages. In order to check this suggestion the α-1,6-glucosidic bond content in the polyglucosides was investigated by means of the periodate oxidation method.

The Type of Glucosidic Bonds in the Polyglucosides

The detailed procedure employed for the determination of periodate utilized and formate released in the oxidation of the polyglucosides has been described in an earlier communication [8]. Periodate was estimated according to Fleury and Lange [9] by reduction with excess of sodium arsenite and back titration of the remaining arsenite with iodine. Formic acid was estimated according to Halsall, Hirst and Jones [10] and modified by Ranken and Jeanes [11] by titration with barium hydroxide after destruction of excess periodate with ethylene glycol. Polyglucoside aliquots (40 to 50 mg) were dissolved in 20 ml of boiled, distilled water, 20 ml of 0.037 M sodium periodate solution was added (the latter was also prepared with boiled, distilled water), and the mixture incubated at 25°. In individual experiments periodate and formic acid were estimated after an incubation time of either 72 or 96 hours. However, earlier observations [8] about the completeness of the reaction at 72 hours were confirmed. The results obtained are presented in Table 2.

It will be seen from Table 2 that during the oxidation of the polyglucosides 0.3-0.5 moles of formic acid was liberated per mole of glucose residues, that is, approximately 5-7 times the amount formed in glycogen oxidation and about one-half to one-third of that formed in the oxidation of dextran. Consequently, 32-47% of the total amount of glucose residues in the molecule are linked by 1,6-bonds, or are terminal. Such a considerable proportion of 1,6-bonds in the molecules suggested that they were either highly branched (one branching point approximately for every two glucose moieties of chain), or else contained a certain proportion of 1,6-bonds in the chains themselves. The quantitative ratios of iodate utilized for formate produced, obtained in the present work, indicated the absence of any appreciable number of 1,3-bonds in the polyglucosides.

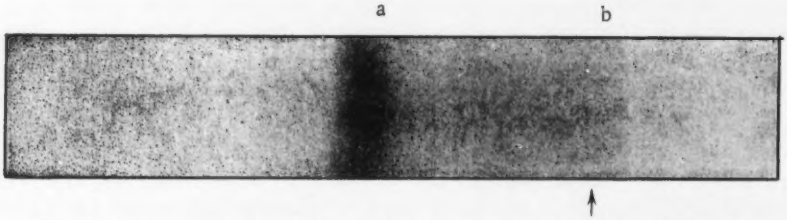

Fig. 2. Electropherogram of liver glycogen. a) Glycogen fraction I; b) glycogen fraction II.

The Molecular Weight of the Polyglucosides

The molecular weight (M) was determined by a chemical method which involved the estimation of reducing power before and after acid hydrolysis, the ratio of the two values affording an indication of the degree of polymerization and therefore the means of calculating M. The actual method employed was as modified by Stepanenko and Kainova [12, 13]. The reducing power of the polysaccharide was determined according to Hagedorn and Jensen and compared with that of the simplest polymer homologue — maltose. The reducing power of maltose was determined for a range of concentrations and a standard curve was constructed from the

values obtained. The value of M was calculated according to the equation: $M = 2\frac{a \times 162}{b}$, where a = polyglucoside concentration, b = its reducing power calculated from the maltose standard, 2) is the coefficient of maltose polymerization (see Table 3).

TABLE 2

Polyglucoside Structure

Preparation	Formic acid formed*	Periodate uptake*	% of 1,6-bonds	% of other bonds
Glycogen	0,07	1,10	7	96
Dextran Macrodex**	0,87	1,87	87	13
Polyglukan***	0,88	1,87	88	11
Polyglucoside				
4.IV 1956	0,32	1,35	32	71
18.V 1956	0,36	1,33	36	61
25.V 1956	0,47	1,52	47	58
11.XII 1956	0,43	1,40	43	54
5.IV 1957	0,37	1,34	37	60
9.VIII 1957	0,33	1,40	33	70

* In moles per mole of glucose residue.

** Swedish dextran preparation.

*** Polish dextran preparation.

TABLE 3

The Molecular Weight of Polyglucosides

Date of preparation	M as determined by chemical methods	M_{SD}
4.IV 1956	24 000	27 000
18.V 1956	7 000	—
25.V 1956	8 000	—
2.VI 1956	13 000	—
11.XII 1956 (*I*)	6 000	~6000 (5 500)
11.XII 1956 (*II*)	30 000	—
5.IV 1957	6 000	—
9.VIII 1957	13 000	—

The values of M for two polyglucoside preparations were also determined on the ultracentrifuge.*

The following data were obtained from the diffusion and sedimentation experiments with the polyglucoside preparation of April 4th, 1956: S = 2.2 Svedberg units, $D = 5 \cdot 10^{-7}$ cm^2/sec. For the preparation of December 11th, 1956, the values were, S = 0.94 Svedberg units, $D = 10.6 \cdot 10^{-7}$ cm^2/sec.

The specific volume was taken as V = 0.61 cm^3/g [19]. Using these data the values of M for the polyglucoside preparations of April 4th, 1956 and December 11th, 1956, were calculated to be 27,000 and about 6,000 (5,500) respectively (see Table 3). The values were means, since the preparations showed a certain degree of polydispersion.

It will be seen (Table 3) that the values for M obtained by sedimentation were in good agreement with those obtained by chemical means. It will also be seen that the value of M in different preparations varied from 6 to 30 thousand.

* Determination of M values on the ultracentrifuge was kindly carried out by V. O. Shpikiter in the laboratory of Professor V. N. Orekhovich of the Institute of Biological and Medical Chemistry, AMN SSSR, the author wishes to express his gratitude for the courtesy.

Enzymatic Hydrolysis of Polyglucosides

As shown in a previous communication [5], the polyglucosides prepared by the "alkaline" or "aqueous" method were frequently hydrolyzed by α-amylase.

Investigations on the effect of α-amylase on the polyglucosides, prepared by the method described in the present communication, showed that the degree to which they were split by the enzyme varied, for individual preparations, between 10 and 20% for a 48-hour incubation. In the same interval, as well as in more prolonged intervals, the effect of β-amylase on the same preparations was nil (Table 4). Thus, the polyglucosides were partially split by α-amylase, and not at all by β-amylase. When the nondialyzed α-amylase digest of the polyglucosides (such preparations would contain a certain proportion of low molecular weight α-dextrins) was treated with β-amylase, 4-5% of the material was attacked by the enzyme.

TABLE 4
The Degree of Polyglucoside Splitting by α- and β-Amylase

Polysaccharide	α-amylase	β-amylase
Glycogen	60—65%	35—45%
Dextran	0	0
Polyglucoside		
14.IV 1956	16	—
18.V 1956	13	0
12.VI 1956	18	0
11.XII 1956	11	0
1.XII 1956	19	0
5.IV 1957	—	0
8.VIII 1957	19	0

All experiments on the α- and β-amylolysis of the polyglucosides were carried out together with corresponding digestions of glycogen. The degree of glycogen hydrolysis which, in individual cases, varied between 60-65% with α-amylase, and 35-40% with β-amylase, was taken as an indication of enzymatic activity. As is well known, dextrans are attacked by neither α- nor β-amylase.

Polyglucoside hydrolysis by amylases was investigated using salivary amylase, filtered and diluted ten-fold, and a 0.4% solution of soybean β-amylase prepared according to Bourne et al. [14]. All β-amylase preparations were checked for purity and for absence of α-amylase activity in separate preliminary experiments.

Paper Electrophoresis of Polyglucosides

Paper electrophoresis was carried out in a plexiglass chamber. The details of the apparatus used were described by Gendon [15].

Samples of the polyglucosides, as well as of glycogen and the various dextrans, were placed on paper strips 41 x 4 cm. Electrophoresis was carried out for 18-20 hrs. using veronal buffer pH 8.6, ionic strength 0.05, under a voltage 200-220 V with a current of 5-6 mA [16]. In some experiments borate buffer was also used. The dried strips were treated with periodate in order to oxidize the polysaccharides, and then with Schiff's reagent. The staining method used was that of Baudoin et al. [17], with slightly altered oxidation and dyeing periods.

The actual procedure was as follows:

1. Periodate solution (sodium or potassium salt): The salt (1 g) was dissolved in a small amount of water, 1 ml of 70% nitric acid (d = 1.42) was added and the volume made to 100 ml with water. This quantity was sufficient for the treatment of 15-20 paper strips, and did not deteriorate on storage in the cold for 1-2 days.

2. The sulfurous acid-fuchsin solution was prepared by grinding 1.5 g of fuchsin base in an agate mortar with a small volume of hot water, washing the solution into a graduated flask with water and adding 1.5 g of potassium metabisulfite, 3 ml of concentrated hydrochloric acid and water to 200 ml. The solution, which decolorized slowly on standing, was left in the cold overnight and filtered. The color appeared transiently during filtration, but disappeared later. The solution, which kept about 10 days, was sufficient for the treatment of 15-20 paper strips.

3. Washing solution: The solution contained 39.0 ml of freshly prepared stock bisulfite solution and 15 ml of concentrated hydrochloric acid in a total volume of 1 liter. The stock bisulfite solution was prepared by passing freshly generated (from sulfuric acid and metallic copper) SO_2 into a 20% solution of sodium carbonate until the solution produced a blue color with congo red.

The dried paper strips were placed for 45 min. into the periodate solution, washed three times with distilled water (5 min per wash) and immersed into the Schiff's reagent for 30 min. The stained papers were then placed into the freshly prepared bisulfite wash for 30 min. The bisulfite treatment was repeated three times, and the papers were dried and stored away from light.

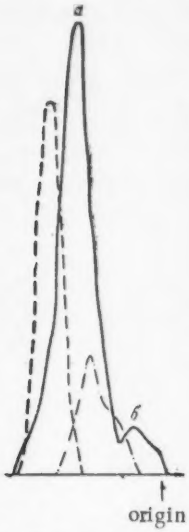

Fig. 3. Electrophoretic resolution of polysaccharides. a) Fraction I; b) fraction II; ———) glycogen; ------) dextran; —•—•—•—) polyglucoside.

It was found that the position of polyglucoside bands in the electropherograms coincided with that of the dextran. Glycogen was resolved into two components. The first component appeared as a light, broad band (Fig. 2, band b), while the second produced a considerably darker band (Fig. 2, band a). The general picture obtained (Fig. 2) was very reminescent of that described by Last [18] for the electrophoresis of liver glycogen in triethylamine monophosphate buffer pH 7, followed by iodine-staining of the paper strips. The position of the polyglucosides and dextrans frequently coincided with that of the darkly staining glycogen band (Fig. 3), but in some experiments the mobilities of the polyglucosides and dextrans were slightly in excess of that of glycogen. As mentioned above, the coloration of the dextrans differed from that of the glycogens in the present work. Glycogens produced a red color, while the dextrans stained purple. The polyglucosides produced colors of lower intensity. The actual colors produced by polyglucosides resembled that of dextrans in some experiments, and of glycogen in others.

DISCUSSION

The hydrolysis of crude liver glycogen by α-amylase produced polyglucosides whose molecular weights ranged from 6,000 to 30,000. Their characteristic property was a high content of α-1,6-bonds (30-40%), and the absence of α-1,3-linkage. It may be assumed that these compounds are α-dextrins, arising from the action of α-amylase on glycogen. However, it appears from published data [20] that prolonged amylolysis of glycogen gives rise to α-dextrins whose polymerization coefficient does not exceed 8-9.

In the present experiments, polysaccharides of the latter category were removed by dialysis. Larger α-dextrins, with a polymerization coefficient of 30 to 150, could not be expected to contain the large proportion of 1,6-bonds found in the present work. Nor would they be expected to be completely resistant to β-amylase. The present results, as well as those of Wolfrom, who isolated isomaltotriose from glycogen hydrolyzates, allow two possible conclusions.

1. Animal livers contain certain polyglucosides which are distinguished by their high content of 1,6-bonds. 2. Glycogen molecules contain sections which differ in their structure from "usual" glycogens in that they contain 1,6-bonds not only in the branching points, but also in the terminal chains. From present results, the possibility still remains that 1,4- and 1,6-bonds alternate throughout the molecules in the ratio of 2:1.

Attempts to separate the polyglucosides from glycogen by paper electrophoresis were not successful. As shown above, the polyglucoside band usually occupied the same position as one of the glycogen bands, namely, the more intensely staining band a (Fig. 2); the dextran band frequently occupied the same positions, although some dextran preparations showed a slightly higher mobility.

Using borate buffer, it was generally possible to resolve dextran into two bands. The various mobilities of dextran fractions during electrophoresis in Tiselius-type apparatus with borate buffer, have been described [21]. However, in the present work clear-cut separation of the dextrans and polyglucosides on paper in borate buffer was not achieved.

It is noteworthy that the method described in the present communication did not always result in a yield of polyglucosides. On many occasions, after the addition of alcohol to the dialyzed amylose digest of glycogen there appeared no precipitate whatever. The authors assume that the optimal conditions (possibly a suitable physiological state of the animal) for the accumulation of large amounts of polyglucosides in the liver have not yet been found.

The problem of their function in the body and the special features of their metabolism is still unsolved, and will be the subject of our further investigations.

SUMMARY

A method for the preparation of polyglucosides from salivary α-amylase digests of glycogens has been described.

The polyglucosides were found to have a molecular weight in the range 6,000 to 30,000, specific rotation in water − 190-200° and a content of 1,6-bonds − about 35-40%. They were hydrolyzed by α-amylase to the extent of about 10-20%, and by β-amylase not at all.

The possibility of the polyglucosides existing in the liver as individual compounds, or comprising portions of some special glycogen molecules, has been discussed.

LITERATURE CITED

[1] E. L. Rozenfel'd, Biokhimiia 21, 84 (1956).

[2] E. L. Rozenfel'd and I. S. Lukomskaia, Biokhimiia 21, 412 (1956).

[3] E. L. Rozenfel'd and I. Lukomskaya, Clin. chim. acta 2, 105 (1957).

[4] M. Wolfrom and A. Thompson, J. Am. Chem. Soc. 78, 4182 (1956).

[5] E. L. Rozenfel'd and I. S. Lukomskaia, Doklady Akad. Nauk SSSR 111, 1058 (1956).

[6] I. S. Lukomskaia, "Dextrane and isomaltose hydrolysis by enzyme preparations from various animal tissues," Thesis, (Moscow, 1958).*

[7] S. Schwimmer and A. Benvenue, Science 123, 543 (1956).

[8] E. L. Rozenfel'd, Biokhimiia 23, 635 (1958).

[9] P. Fleury and J. Lange, J. Pharm. Chim. 17, 196 (1933).

[10] T. Halsall, E. Hirst, and I. Jones, J. Chem. Soc. 1599 (1947).

[11] I. Rankin and I. Jeanes, J. Am. Chem. Soc. 77, 4435 (1954).

[12] B. N. Stepanenko and A. S. Kainova, Doklady Akad. Nauk SSSR 95, 1263 (1954).

[13] A. S. Kainova, "The enzymatic synthesis of glycogens with a study of synthesized glycogens," Thesis, (Moscow, 1954).*

[14] E. Bourne, I. Macey, and V. Peat, J. Chem. Soc. 877 (1945).

[15] Iu. Z. Gendon, Zhur. Mikrobiol. Epidemiol. i Immunobiol. 9, 91 (1956).

[16] A. E. Gurvich, Lab. Delo 1, 3 (1955).

[17] A. Baudoin, J. Levin, and P. Hillion, C. r. Soc. biol. 148, 1033 (1954).

[18] F. Bertrand and L. Last, Bioch. Z. 327, 354 (1956).

[19] A. Ingelman and E. Holting, Ark. kemi 1, 61 (1949).

[20] K. Myrback and E. Villstaedt, Ark. kemi 7, 403 (1954).

[21] K. Zakrzensky, Z. Maj, and K. Murawsky, Biokhimiia 21, 596 (1956).

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* In Russian.

A CHROMATOGRAPHIC METHOD FOR DETERMINATION OF 4(5)-AMINOIMIDAZOLE-5(4)-CARBOXAMIDE AND THE CONTENT OF THIS COMPOUND IN HUMAN AND ANIMAL URINE

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Earlier, one of the authors [1] showed for the first time the presence in healthy human urine of the intermediate of urine metabolism, aminoimidazolecarboxamide (AICA, 4(5)-amino-5(4)-imidazolecarboxamide). We also showed that in paper chromatography, the behavior of synthetic AICA, obtained by the method of Shaw and Wooley [2], and the product isolated from urine agreed completely, which confirmed its identity (Figure). Up to now, the presence of AICA in the tissues and fluids of animals has not been shown and its presence in urine was considered unlikely [3].

Fig. Position of the blue AICA spot on the chromatogram of bases extracted from urine. 1) Extract from 2 ml of urine (R_f = 0.35); 2) the same + 1.2 mg of AICA (R_f = 0.35); 3) 1.2 mg of AICA (R_f = 0.30).

Accumulation of AICA and AICA-ribose-5-phosphoric acid (AICA-ribotide) has been found in bacterial cultures when they grow in the presence of sulfonamides, and also in cultures of mutant bacteria which cannot synthesize purines. By use of the isotope method it has been shown that AICA is transformed in microorganisms and in animal bodies into purines of nucleic acids, into uric acid, and into allantoin. It has also been shown that in animal tissues and microorganisms the enzymatic condensation of AICA-ribotide gives with formyl residues hypoxanthine ribotide (inosinic acid). This transformation is reversible; it occurs with participation of a formylating coenzyme (tetrahydrofolic acid) [4].

The observation of the constant presence of AICA in urine raises the question of the physiological norm of its excretion in humans and animals and also the possibility of a change in the excretion of AICA in pathological conditions connected with injury to purine metabolism, such as leucosis, gout, radiation sickness, medicinal use of antimetabolites of purine metabolism, etc.

In the present work we give data on the amount of the excretion of AICA in healthy humans and animals (and also in rats after injecting them with the folic acid antagonist, aminopterine), obtained by our chromatographic method for the quantitative determination of AICA.

METHOD

Principle of the method. The quantitative determination of AICA in urine was carried out by spectrophotometric estimation of the blue color developed on the chromatogram of the bases isolated from urine by treating the AICA spot directly with a solution of diazobenzenesulfonic acid (Pauly reagent). The use of dilute Pauly reagent permits a considerable weakening in pigment formation (red and yellow) from other diazo positive substances of urine. AICA was fully removed by n-butanol from a pure, saturated salt solution. When AICA

was added to urine and extracted in the same way there was a considerable loss, reaching 40-50%. The size of the loss depended on the individual composition and concentration of the urine; it could be somewhat decreased by preliminary treatment of the acidified urine with ether. When AICA was extracted continuously from urine by n-butanol for 8-10 hours, the loss of AICA did not decrease, but on the contrary, increased. Evidently it depends on the presence in urine of some substance which can decompose AICA during its extraction.

When the analysis was carried out under strictly determined conditions, the amount of AICA loss in a given sample of urine was proportional to the concentration of AICA (in definite limits) and was the same in parallel tests.

This permits the use of a correction for the incomplete removal of AICA in urine: the so-called method of the "internal standard", found by adding to parallel samples of the urine under investigation of definite amounts of synthetic AICA.

Carrying out the determination. The daily urine was collected in a vessel with 2-3 ml of concentrated HCl or glacial acetic acid and brought to a definite volume. A measured portion of the urine (about 50 ml) was extracted for 10 min with freshly distilled ether which was freed from peroxides with $FeCl_3$. After removal of the ether layer, the aqueous phase was neutralized with dry Na_2CO_3 and 4 samples of 10 ml of urine were placed in dark bottles (volume 50 ml) with ground glass stoppers; to each was added 2 g of a salt mixture consisting of 6 parts of anhydrous Na_2SO_4 and 1 part of $Na_3PO_4 \cdot H_2O$, and 10 ml of n-butanol. To two samples was added 0.1 ml of standard AICA solution in 50% alcohol (6 mg in 100 ml). Air was forced out of all the bottles by a stream of nitrogen from a cylinder for 10 min, then they were rapidly covered and shaken for 30 min. The n-butanol layer was separated by centrifuging and sucked off. The water phase was extracted a second time in the same way with an equal volume of n-butanol. The combined alcoholic phases were transferred to a separatory funnel and shaken three times for 10 min each with 0.5 N HCl (5 + 3 + 3 ml); the aqueous layer separated well by settling. The combined aqueous fractions, which contained the AICA, were evaporated dry in a round bottomed flask under vacuum in a nitrogen atmosphere. The residue was dissolved in 0.2 ml of methyl alcohol (the flask was cooled with ice and tightly covered with a plastic stopper to prevent evaporation of the alcohol) and transferred to a small test tube (5 × 40 mm) with a plastic stopper. Using a micropipette, 40 μl of each extract, which corresponded to 2 ml of urine with 1.2 μg of added AICA, was placed on the chromatographic paper (Whatman No. 1 or No. 2; Leningrad chromatographic paper was also suitable if it was first carefully washed with a 0.5% solution of EDTA [5] and dried); standard AICA was also added as an indicator. As a solvent we used n-butanol saturated with a 10% ammonia solution; the experiment lasted 16-18 hours. Then the chromatogram was dried at room temperature and the position of the AICA spot, which absorbed ultraviolet light, was determined with a Brumberg ultrachemoscope. The strip with the AICA spot, cut from the chromatogram, was developed by passing it through a cuvette with a cooled solution of diazotized sulfanilic acid, the excess reagent was removed, by placing the chromatogram between sheets of filter paper, and the color was at once fixed by washing in 96% alcohol, after which the chromatogram was air dried.

The diazo reagent was prepared as follows: 4.5 g of sulfanilic acid was dissolved in 45 ml of 12 N HCl with heating and addition of water to 500 ml. 10 ml of this solution was cooled in ice, mixed with 10 ml of a 4.5% solution of $NaNO_2$, and allowed to stand in the cold for 15 min. This solution can be kept in the refrigerator for 3 days. Before use, 1 volume of this solution was mixed with 1 volume of a 20% Na_2CO_3 solution and 6 parts of ice from distilled water.

The blue spot from AICA, cut from the chromatogram, was extracted with 3 ml of 10% ammonia solution. The extract was filtered and its optical density was determined at 570 mμ in a cuvette 1 cm thick in a Beckman type spectrophotometer (SF-4).

The AICA content in the urine was calculated by the formula $\frac{a\,c}{(b-a)\cdot d}$ = number of μg of AICA in 1 ml of the urine studied, where a is the reading on the spectrophotometer for the sample of urine, b is the reading on the spectrophotometer for the sample of urine with the added standard AICA solution, c is the amount of added AICA in the sample, added on the chromatogram (1.2 μg), d is the volume of urine which corresponds to the amount placed on the chromatogram (2 ml).

This method permits determination of 1 μg of AICA with an accuracy on the order of ±10% (with larger amounts, the accuracy increases).

AICA Content in the Urine of Healthy Humans and Animals

When the AICA content was determined by this method in the daily urine of 10 healthy humans, it was found that extraction of AICA varied from 0.6 to 1.5 mg in 24 hours, or from 9 to 20 μg per kg of body weight in 24 hours (Table 1); in children during their first year, the excretion of AICA per unit weight was higher.

TABLE 1
Excretion of AICA in the Urine of Healthy Humans in 1 Day

Sex	Age	AICA in 24 hrs	
		total amt in mg	μg per kg body wt
f	18 yrs	0,7	14,5
f	29 "	1,5	20,0
f	29 "	1,5	20,0
f	55 "	1,05	14,4
f	55 "	1,2	16,0
f	56 "	0,65	9,0
f	80 "	0,9	16,0
f	83 "	0,6	10,0
m	29 "	1,4	18,0
m	60 "	0,7	10,0
m	3 weeks	0,3	80,0
m	6 "	0,5	110,0
f	3 yrs	0,52	32,3
Limits of variation (in adults)		0,6—1,5	9—20

TABLE 2
Excretion of AICA in the Urine of Several Laboratory Animals

Type of animal	Body wt. in kg	AICA in μg in 24 hrs	
		total amount	per kg body wt
rat	0,300	19,8	66
"	0,300	20,0	66
"	0,085	12,0	140
" (stored)	0,085	11,0	131
rabbit	2,65	114	43
"	2,65	122	46
"	2,65	103	40
dog	18,000	900	50

We also studied the excretion of AICA in rats, rabbits, and dogs (Table 2). Young rats excreted more AICA per kg of body weight than did more mature rats. When we repeated the AICA determination on the same animal, the variation was slight, and one day starvation had no effect on AICA excretion.

To determine the role of the intestinal flora as possible sources of AICA formation in the animal organism, we added terramycin (100 mg per day) and sulfaguanidine (0.5 g per day) to the ration of the rats for 72 hours before the experiment and at the time of collecting the urine, which according to the literature, results in almost complete intestinal sterilization [6]. Although under these conditions there was a considerable decrease in diuresis, the daily excretion of AICA remained almost unchanged.

TABLE 3
Effect of a Subcutaneous Injection of Aminopterine on the Excretion of AICA in Rat Urine

Expt No.	Wt. of rats	Date	Aminopterine, μg/kg	AICA μg/24 hrs
1	300 g	17.6	—	20
		26/6	—	18
		3/7	160	68
		4.7	—	23
		12/7	160	66
		13/7	160	64
2	200 g	7/I	—	17
		15/I	125	98
3	200 g	7/I	—	17,5
		15/I	125	68
4	255 g	13/II	—	20
		19/II	480	58
5	325 g	13/II	—	23
		19,II	380	50

This shows that the intestinal bacteria do not play an important part in the formation of AICA of the urine, and confirms its endogenous origin.

The chief source of AICA formation in the animal organism is evidently the enzymatic splitting of AICA-ribotide [7], which is an intermediate link in the biosynthesis of inosinic acid, and perhaps in the breakdown of purine nucleotides.

After a single subcutaneous injection in rats of the folic acid antagonist, aminopterine (6-aminopteroylglutamic acid) in doses of 125-480 μg per kg of body weight, we observed a considerable increase in amount of AICA in the urine (Table 3) which agrees with the idea of the participation of the formylating coenzyme (H_4-folic acid) in the conversion of AICA ribotide into inosinic acid.

SUMMARY

We have worked out a method for quantitative determination of AICA in urine based on paper

chromatography of a butanol extract of urine and spectrophotometric estimation of the blue color developed in the reaction of AICA with diazobenzenesulfonic acid.

We have established the normal limits of AICA in urine of healthy humans (0.6-1.5 mg AICA in 24 hours) and also the values for the excretion of AICA in rats, rabbits, and dogs. The excretion of AICA in rat urine is not decreased when terramycin and sulfaguanidine are added for the intestinal flora; it is considerably raised after a single subcutaneous injection of aminopterine.

LITERATURE CITED

[1] G. Ia. Vilenkina, Vopr. Med. Khimii 2, 450 (1956).

[2] E. Shaw and D. W. Woolley, J. Biol. Chem. 181, 89 (1949).

[3] J. Scegmiller et al. J. Biol. Chem. 216, 653 (1956).

[4] E. F. Efimochkina and A. A. Poznanskaia, Vopr. Med. Khimii 3, 243 (1957).

[5] T. V. Venkstern and A. A. Baev, Biokhimiia 22, 1043 (1957).

[6] E. Schram and R. Crokaert, Biochim. biophys. acta 26, 300 (1957).

[7] J. Flaks et al. Biol. Chem. 228, 201 (1957).

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LIPOXIDASE IN THE ROOTS OF THE SQUASH (CUCURBITA PEPO L.)

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Since the discovery of lipoxidase in 1932, a whole series of experimental and review papers have been published which discuss the investigation of this enzyme.

The chief objects of study have been the enzyme itself, the specificity of its substrates, the effects of inhibitors, and methods of isolating and purifying it. According to the data of Franke [1], the best substrates for lipoxidase are linoleic or linolenic acids at pH 9.4. The enzyme is usually studied at pH 6.8; in this case its activity is about 40% of the maximum. Lipoxidase is an enzyme without a prosthetic group, containing no metals and is insensitive to cyanide or NaN_3 (10^{-3} M).

Freshe and Franke [2] recommend the investigation of lipoxidase in tissue homogenates in the presence of inhibitors of cytochrome oxidase to exclude the possibility of the participation of the cytochrome system in the oxidation of linoleic acid. In experiments with lipoxidase isolated from soy beans it was shown that its activity was depressed by substances of the type of phenols which reduced the oxidation-reduction potential. A number of other studies were concerned with the question of the distribution of lipoxidase and its localization. van Fleet [3] showed the presence of lipoxidase in tissues which contained oil and also related its activity to the formation of seeds. However, Sŭllmann [4] had already established the presence of lipoxidase in leaves and roots of different families of beans and also in the leaves of solanaceae and labiatae. Kolesnikov [5] observed lipoxidase in the leaves of barley. Budnitskaia [6] found it in couch grass, begonia, and clover flowers. Sisakian and Kobiakova [7] showed the localization of the enzyme in the leaf plastids and storage organs of beets and carrots. Evidently the value of lipoxidase is not limited to oxidation of unsaturated fatty acids; it can oxidize directly a number of other substances which share in metabolism, for example, amino acids, according to Mikhlin and Pshenova [8]. In our experiments on the study of the lack of some ions (Ca, K, NO_3, PO_4) in the nutrient medium during respiration of homogenates of the root of the squash, it was shown that especially in the absence of Ca the culture behaved differently in the suppression of O_2 consumption by malonate (5×10^{-2} M) and NaN_3 (10^{-3} M) in the presence of succinate.

We assumed that in plants cultured in solutions which did not contain Ca, oxidizing systems developed in greater degree and were insensitive to NaN_3, but in homogenates of these plants we did not find riboflavin, and we assumed that under these conditions there might be a change in lipoxidase activity.

METHODS

Squash (Cucurbita pepo L.) were grown in a 350 ml vessel for 7 days on a nutrient medium with the following composition: 4.87 $Ca(NO_3)_2$; 1.47 KH_2PO_4; 1.98 KNO_3; 0.81 $MgSO_4 \cdot 7H_2O$; 1.34 KCl and 0.062 $FeCl_3$ (in μ moles), and in solutions in which Ca and K were replaced by Na, and NO_3 and PO_4 by chloride. During growth the solutions were not renewed and only distilled water was added to the vessel. In each vessel we set out 20 clean sprouting plants. The cultures were kept at room temperature in daylight.

The entire root system of the plants was first grated and then homogenized in a glass homogenizer without adding liquid and at 0°. Lipoxidase activity was determined in a Warburg apparatus. In the main vessel

we placed 1.5 ml of homogenate, 1.0 ml of 0.15 M phosphate buffer, pH 7.0, 0.5 ml of distilled water or $8 \cdot 10^{-3}$ M NaN_3. In the side arm we placed 1 ml of 0.1 M Na linoleate, pH 7.0 (linoleic acid was dissolved in 0.1 N NaOH and neutralized with HCl). In the central section we placed 0.2 ml of 30% NaOH; temperature of incubation 26°.

The basal respiration was determined over 40 min at 10 min intervals, then the substrate was added from the side arm and the change in O_2 consumption was determined. At the end of the incubation we measured the pH of the sample; because of differences in the acidity of the homogenates it varied from 6.0 to 6.3. The nitrogen content of the homogenate was determined by the Kjehldahl method at the end of incubation. The value of Q_{O_2} was calculated in μl O_2/mg N/10 min.

RESULTS

The course of oxygen absorption by the root homogenates grown in full nutrient solutions and in nutrient solutions without Ca (without addition of NaN_3) is shown in the figure. This shows that the consumption of oxygen in 20-30 min after the beginning of incubation ceases to vary; we take this level as the value of the basal respiration. After addition of linoleic acid during 30 min the oxygen consumption is linear.

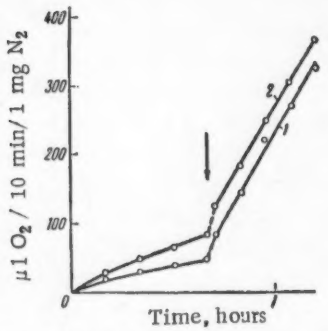

Fig. O_2 consumption in squash root homogenate and its change after addition of Na linoleate (denoted by the arrow).
1) Nutrient solution, 2) nutrient solution without Ca.

The results of the determination of lipoxidase activity are given in the Table. One group of plants was grown under conditions of daily illumination on complete nutrient solution and on the incomplete solution, which lacked Ca, K, NO_3, and PO_4 ions, while the other group of plants was kept for 3 days in the dark. Evidently, such a short period of absence of light did not affect the activity of the oxidases under the experimental conditions. Azide in concentrations of 10^{-3} M suppresses endogenous respiration in the absence of sodium linoleate; in plants grown in the light, by 47%; in plants grown in the dark, by 52%; and in plants grown without Ca, by 30%. The consumption of oxygen (calculated per mg of nitrogen) was highest in homogenates from plants grown without Ca (both in the presence of NaN_3 and without it). After addition of Na linoleate, the difference between Q_{O_2} in the different variants vanished.

In experiments in which we studied the effect of a substance of the polyphenol type, Na gallate, on root respiration, we used the whole top of the squash root with a length of 1.5-2 cm, and the homogenate of roots grown in the absence of NO_3. Respiration was determined at pH 5.2 (the optimum for polyphenol oxidase) without adding substrate. After 40 min from the beginning of incubation, we added 1 ml of 1% Na gallate, pH 5.0, to 2 ml of the main suspension. Thirty min before beginning the experiment, the roots were cut up with scissors and put into distilled water at room temperature, then 50 pieces were transferred to the vessel.

The consumption of oxygen by the roots after addition of Na gallate was almost unchanged, but the respiration of the homogenate fell by about 70%.

DISCUSSION OF RESULTS

From these results it is evident that in squash roots, which have neither green assimilating organs nor storage organs for holding fats, lipoxidase is present. Perhaps because of the peculiarity of roots as distinct from leaves, lack of light does not affect the lipoxidase activity of roots, while, according to the data of Sisakian and Kobiakova [7], in beet leaves light permits the formation of lipoxidase.

It is still not clear what is the function of lipoxidase in squash roots after culturing for a week, when the cotyledons are already functioning as assimilating organs, and it would be difficult to connect the lipoxidase activity with the cost of storing fats in the cotyledons. The experiments with addition of Na gallate to the homogenate without substrate (in endogenous respiration) indicate the participation of lipoxidase also in endogenous respiration. It is true that gallic acid has an inhibiting action on the cytochrome oxidase system – the

percent suppression of respiration by gallate is considerably higher than that which could depend on suppression by oxidases insensitive to NaN_3 (about 50%).

TABLE

Activity of Oxidase in Homogenates from Squash Roots Cultured in Different Nutrient Solutions (values given in μl/mg N/10 min)

Experimental conditions	without NaN_3		with NaN_3	
	without substrate	linoleate	without substrate	linoleate
Nutrient solution, light	7,6	51,5	3,7	51,5
	7,2	62	4,2	52
	—	—	3,45	58
Nutrient solution, dark	7,15	58,5	3,45	62,5
	7,25	58	3,45	57,5
Nutrient solution	13,65	50	8,8	48,3
without Ca	12,1	53,5	9,0	58
without K	—	—	4,75	55
	—	—	5,5	82,5
without NO_3	—	—	3,35	45,75
	—	—	4,15	46
without PO_4	—	—	3,85	56,5
	—	—	4,05	61

According to the data of Oparin and Shubert [9], in such typical "polyphenol oxidase" plants as tea, for example, a leaf suspension after addition of polyphenol gives a higher Q_{O_2} compared to endogenous respiration. We can believe that, as distinct from "polyphenol oxidase" plants, in squash the role of the terminal oxidase is fulfilled especially by lipoxidase.

This is not surprising, since like soy and other plants, it is an oil producer. It is impossible to understand why the inhibiting effect of Na gallate does not appear in the cut-up roots. It is unlikely that this can be explained only by the difficulty of penetration of gallate into undamaged cells. It is possible that the reason here must be sought in a change in enzyme systems from the homogenization.

SUMMARY

In squash root homogenates cultured for 7 days in different nutrient solutions, considerable lipoxidase activity has been found. Lack of Ca, K, N, or P in the nutrient solution or three days of darkness during growth do not affect the lipoxidase activity. A direct connection has not been established between lipoxidase activity and endogenous respiration. The lipoxidase activity is not decreased in the presence of 10^{-3} M NaN_3.

LITERATURE CITED

[1] W. Franke, Erg. Enzymforschung 12, 89 (1951).

[2] H. Freshe and W. Franke, Fette. Seifen. Anstrichmittel 58, 403 (1956).

[3] D. S. van Fleet , Erg. Enzymforschung 12, 172 (1951).

[4] H. Sŭllmann, Experientia 1, 323 (1945).

[5] P. A. Kolesnikov, Doklady Akad. Nauk SSSR 71, 1085 (1950).

[6] E. V. Budnitskaia, Biokhimiia 20, 614 (1955).

[7] N. M. Sisakian and A. M. Kobiakova, Biokhimiia 22, 516 (1957).

[8] D. M. Mikhlin and K. V. Pshenova, Biokhimiia 14, 2, 141 (1949).

[9] A. I. Oparin and T. A. Shubert, Biokhimiia chainago proizvodstva 6, 82 (1950).

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THE REVERSIBILITY OF ACYLASE I ACTION. ENZYMATIC HYDROLYSIS AND SYNTHESIS OF L-ACETYLALANINE

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As is known, animal livers and kidneys are sites of acetylation and transacetylation of L-amino acids [1-5]. The thus formed L-acetylamino acids do not accumulate in the organism, but undergo further transformations. Recently Tallan [6] found L-acetylaspartic acid in animal brain tissue and isolated it from this.

However, until now there has not been sufficient study of how the enzyme systems carry out acetylations and transacetylations of amino acids. Almost nothing is known of the significance of this process in the life activities of the organism.

Spirichev and Shchukina [7], in experiments with acetone treated powder from pig kidney which contained the enzyme acylase I, found that at high concentrations of L-acetylalanine, hydrolysis of the L-form did not go to completion. Also by incubating DL-alanine with the acetone treated powder in the presence of excess sodium acetate they succeeded in carrying out an asymmetric synthesis of L-acetylalanine, which was isolated in a yield of 10-15% of the L-form from the starting DL-alanine.

This gave a reason for assuming that enzymatic hydrolysis of L-acetylalanine by acylase is a reversible process:

$$\text{L-}CH_3CH(NHCOCH_3)COOH \underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}} \text{L-}CH_3CH(NH_2)COOH + CH_3COOH$$

To confirm this assumption we have carried out the asymmetric synthesis of L-acetylalanine from DL-alanine and sodium acetate under the action of pure acylase I, prepared by the method of Ch'i Cheng-wu and Orekhovich [8, 9].

In the present paper we give the results of a study of the equilibrium between the processes of hydrolysis and synthesis of L-acetylalanine by acylase in the presence of different concentrations of sodium acetate. We have also described an enzymatic method of obtaining L-acetylalanine.

EXPERIMENTAL

Hydrolysis of L-Acetylalanine

The hydrolysis was carried out in 0.2 M phosphate buffer, pH 7.2, with different concentrations of sodium acetate (from 0 to 2 M). The concentration of substrate in all cases was 0.5 M, calculated on the L-acetylalanine (in the experiments we used 1.0 M DL-acetylalanine). To each sample we added a dry preparation of purified acylase I (0.3 mg/ml), and the samples were incubated in a thermostat at 38°. The amount of L-alanine formed by hydrolysis was determined according to Van Slyke.

The Figure (curve 1) shows that even in the absence of excess sodium acetate hydrolysis of L-acetylalanine occurs only to the extent of 94%. With increase in the initial concentration of sodium acetate to 2.0 M (curve 4) equilibrium has already been established when 80% hydrolysis is reached.

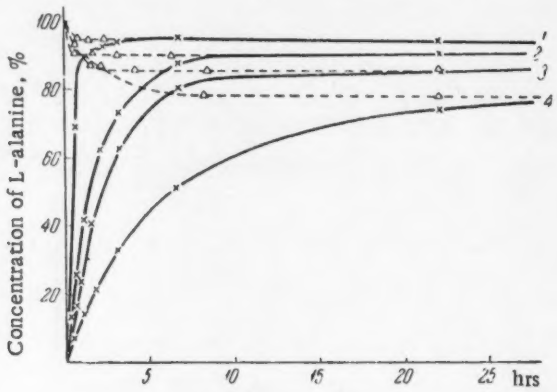

Figure. Hydrolysis and synthesis of L-acetylalanine by the action of acylase I. Initial concentration of sodium acetate in the hydrolysis and synthesis corresponding to curves: 1) 0.0 and 0.5 M; 2) 0.5 and 1.0 M; 3) 1.0 and 1.5 M; 4) 2.0 and 2.5 M.

For all the experiments we calculated the equilibrium constant $K = \frac{[\text{L-acetylalanine}]}{[\text{AcONa}]\ [\text{L-alanine}]}$. The results are given in the Table.

TABLE

Value of the Equilibrium Constants in the Reversible Processes of Hydrolysis and Synthesis of L-Acetylalanine

Hydrolysis						Synthesis					
Initial concentration, M		Final concentration M			equilibrium const	initial concentration, M		final concentration M			Equilib constant
L-acetyl-alanine	AcONa	L-acetyl alanine	L-alanine	AcONa	K_1	L-alanine	AcONa	L-acetyl alanine	L-alanine	AcONa	K_2
0,5	—	0,025	0,475	0,475	0,110	0,5	0,5	0,025	0,475	0,475	0,110
0,5	0,5	0,045	0,455	0,955	0,105	0,5	1,0	0,047	0,453	0,953	0,109
0,5	1,0	0,070	0,430	1,430	0,114	0,5	1,5	0,072	0,428	1,428	0,118
0,5	2,0	0,103	0,397	2,397	0,108	0,5	2,5	0,103	0,397	2,397	0,108

Synthesis of L-Acetylalanine

If the equilibrium constants calculated in the experiments on the hydrolysis of L-acetylalanine reflect the true equilibrium position, then we should obtain analogous results also in the synthesis of L-acetylalanine.

The synthesis of L-acetylalanine was carried out in 0.2 M phosphate buffer, pH 7.2 with sodium acetate concentrations from 0.5 to 2.5 M. Such initial concentrations of acetate guarantee that when equilibrium is reached, the concentrations will equal those which existed in the experiments on hydrolysis. The initial concentration of L-alanine was 0.5 M (as in the experiments on hydrolysis, we used racemic DL-alanine in concentrations of 1.0 M). In order to attain equilibrium more rapidly, we increased the concentration of acylase I in these experiments to 0.7 mg/ml.

The resulting data are given in the figure and the table.

It follows from these results that the values for the equilibrium constant calculated in the experiments on hydrolysis (K_1) and in the experiments on the synthesis of L-acetylalanine (K_2) agree well enough with each other. This indicates that both in the hydrolysis of L-acetylalanine and its synthesis equilibrium is established. This equilibrium position naturally depends on the concentration of the interacting substances. In the experiments with low concentration of sodium acetate, hydrolysis of the acetylamino acid predominates, and with increased concentration of sodium acetate there is a shift in the equilibrium to the side of synthesis. The maximum concentration of sodium acetate in our experiments was 3.5 M. In this case, 28-30% synthesis took place.

Effect of Different Factors on the Enzymatic Synthesis of L-Acetylalamine

Stedman et al. [10] showed that introduction of KCN in the cells of Clostridium Kluyveri caused splitting of acetylphosphate with simultaneous acetylation of amino acids. Lipmann et al. [11] used instead of acetylphosphate, ATP and coenzyme A, and also found acetylation of amino acids with extracts of Escherichia coli.

In view of these facts, we studied the effect of ATP (5 mg/ml) and KCN (0.02 M) on the synthesis of L-acetylalanine in our system. The results showed that the addition of these substances in vitro had no particular action on the synthesis of L-acetylalanine by acylase I. Hence, the enzymatic synthesis of L-acetylalanine is the result solely of the reversible action of acylase I and there is no need for any supplementary factors.

We have shown that the optimum pH for the synthesis of L-acetylalanine is 7.0 to 7.5, which corresponds to the optimum pH for its hydrolysis which was established earlier [12]. At pH below 5 and above 9 the synthesis of L-acetylalanine practically ceases.

Preparative Method for Obtaining L-Acetylalanine

One mole of racemic alanine and 3.5 moles of sodium acetate were dissolved in distilled water heated to 40-50°; the volume of the solution was brought to 1 liter. The pH of the solution was brought to about 7.5 by addition of several ml of acetic acid. The solution was cooled to 37° and 1.0 g of a preparation of dry, purified acylase I was added.

The mixture was incubated in a thermostat at 37° for 24 hours. Equilibrium was reached in this time.

To isolate the L-acetylalanine, we acidified the incubation mixture with acetic acid to pH 5.0 and evaporated it to small volume in a vacuum at 25-30°. The residue was treated with 500 ml of absolute alcohol. After standing for several hours at 0-3°, the precipitate of unreacted amino acid was filtered off. The alcoholic filtrate was acidified with concentrated HCl to pH 1-2 and evaporated dry in a vacuum at 20-25°. Water was added to the dry residue to remove HCl and CH_3COOH and it was again evaporated dry (the operation was repeated twice). Then the residue was dissolved in the minimum amount of water and extracted 10 times with ethyl acetate. The combined extracts were dried with Na_2SO_4 and evaporated to small volume. The resulting crystalline residue of L-acetylalanine was recrystallized from acetone. The yield of pure L-acetylalanine was 6.5 g or 15% of the L-form of the starting DL-alanine. For analysis the substance was further recrystallized from water and dried over P_2O_5 in a vacuum at 75°, m. p. 126°, $[\alpha]_G^{22} = -66°$ (2% solution in water, l = 2) [13].

Found %: C 45.76; H 7.09; N 10.61. $C_5H_9O_3N$. Calculated %: C 45.75; H 6.92; N 10.68.

DISCUSSION

The previous data show that synthesis of L-acetylalanine occurs in appreciable amount only when the sodium acetate concentration is above 0.5 M. However, we can assume that under the conditions of the live, undamaged cell the rapid removal of the acetylamino acid which is formed will permit a shift in the equilibrium to the side of synthesis. In a recent work of Phillips and Anker [3] the possibility of acetylating cyclohexylalanine with a preparation from rat kidney was shown. The authors showed that the amount of acetylcyclohexylalanine which was formed in kidney slices was 100 times greater than the amount of the cyclohexylalanine which was formed by aqueous extracts of the kidney which did not contain cells. The authors assumed that this process

occurred as a result of the action of acylase I which was contained in their preparation. This viewpoint is confirmed by our data on the synthesis of L-acetylalanine by the action of pure acylase I. According to our preliminary results, acylase I can carry out the synthesis of a whole series of other acetylamino acids. Since the bond between the acetyl group and the amino acids resembles a peptide link and dipeptides are hydrolyzed by acylase I [14-16], it is possible to assume that acylase I can play an important part in the biosynthesis of peptides.

SUMMARY

We have studied the action of pure acylase I on L-alanine and L-acetylalanine in the presence of different amounts of sodium acetate. We have shown that acylase catalyzes the reverse processes of synthesis and hydrolysis of L-acetylalanine.

We have calculated the equilibrium constant of this process. Its average value is 0.11.

We have described a preparative method of enzymatic synthesis of L-acetylalanine from DL-alanine and sodium acetate by the action of acylase I. The yield of pure L-acetylalanine is 15% based on the L-form of the starting DL-alanine.

LITERATURE CITED

[1] K. Bloch and E. Borek, J. Biol. Chem. 164, 483 (1946).

[2] N. R. Gutmann and J. L. Wood, J. Biol. Chem. 189, 473 (1951).

[3] M. B. Phillips and H. S. Anker, J. Biol. Chem. 227, 465 (1957).

[4] K. Bloch and D. Rittenberg, J. Biol. Chem. 169, 467 (1947).

[5] T. C. Chou and F. Lipmann, J. Biol. Chem. 196, 89 (1952).

[6] H. H. Tallan, J. Biol. Chem. 224, 41 (1957).

[7] V. B. Spirichev and L. A. Shchukina, Zh. Obshchei Khim. 28, 1709 (1958).

[8] Ch'i Cheng-wu and V. N. Orekhovich, Biokhimiia 22, 838 (1957).

[9] Ch'i Cheng-wu and V. N. Orekhovich, Biokhimiia 23, 772 (1958).

[10] E. R. Stadman, J. Katz, and H. A. Barker, J. Biol. Chem. 195, 779 (1952).

[11] W. K. Maas, G. D. Novelli, and F. Lipmann, Proc. Nat. Acad. Sci. 39, 1004 (1953).

[12] Methods in Enzymology, Vol. II, p. 115, Md. Nat. Inst. Health, Bethesda (1955).

[13] S. M. Birnbaum, L. Levintow, R. B. Kingsley, and J. P. Greenstein, J. Biol. Chem. 194, 455 (1952).

[14] S. C. J. Fu, S. M. Birnbaum, and J. P. Greenstein, J. Am. Chem. Soc. 76, 6054 (1954).

[15] K. R. Rao, S. M. Birnbaum, R. B. Kingsley, and J. P. Greenstein, J. Biol. Chem. 198, 507 (1952).

[16] K. R. Rao, S. M. Birnbaum, and J. P. Greenstein, J. Biol. Chem. 203, 1 (1953).

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MUSCLE AMYLASE OF FISHES

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It has previously been assumed without sufficient basis that the breakdown (i.e. "mobilization") of glycogen in the muscles of fishes is carried out entirely by phosphorylation. However, the work of Petrova [1, 2] with extracts of the skeletal muscles of adult rabbits and also the work of Drozdov and Zhuravskaia [3] on the muscles of cattle showed that in muscles, besides the phosphorylytic pathway, there is another method of breakdown, the hydrolytic. We set ourselves the problem of determining whether there is hydrolytic breakdown of glycogen in fish muscles.

The existing literature on fish amylases relates entirely to the amylases of the digestive organs and some of the work is of little value, since it was carried out under conditions which did not exclude the effects of phosphorylating systems. The question is also of general interest and has industrial significance for an understanding of the biochemical processes which occur in the treatment and keeping of fish.

In this work we have studied the muscles of different fish. The investigation was carried out with dialyzed aqueous extracts from various fish muscles prepared under conditions which prevented the growth of microorganisms. Both fresh water and salt water fish were studied.

METHODS

The fish heads were cut off and the skeletal muscle was separated out. Forty g of muscle, after treatment with twice the volume of water and 0.5 ml of toluene as an antiseptic, was carefully ground in a mortar for 30 min. The resulting paste was autolyzed at room temperature for 5-6 hours and then centrifuged. 50 ml of the centrifugate was dialyzed for 24 hours against twice distilled water at 5-8° to remove inorganic phosphates and the components of the adenylic acid system. We used a modified Kritskii apparatus [4] for the dialysis. The resulting dialysate was added to 100 ml of distilled water and centrifuged to remove the globulins which precipitated during the dialysis; the resulting clear dialyzed extract was used for the investigation.

The amylytic activity of the extract was determined by the hydrolysis of starch in samples of the extract to the disappearance of color with iodine and the appearance of reducing substances, determined by the method of Hagedorn and Jensen.

Experiments were carried out without adding buffer solutions, since the pH in the experimental mixtures after dialysis was 6.7-6.8, which is favorable for the action of animal amylases [5]. The pH was measured electrometrically and did not change during the experiment.

We used samples with the following composition to establish the amylytic activity of fish muscles: 20 ml of dialyzed aqueous extract, 3 ml of 1% starch solution, and 0.1 g NaCl as activator [5]. Parallel determinations were carried out in samples with the same composition, but in one case without NaCl and in another with a boiled extract. The samples were incubated 2-3 hours at 37°, and during this time changes in color with iodine and content of reducing substances were determined periodically.

RESULTS

In Table 1 we give the results of the experiments which show positively a definite and apparently considerable amylytic activity in the aqueous extracts of fish muscle. As is evident from the Table, in the absence of NaCl enzymatic splitting of starch occurs very slowly, from which it follows that for the action of fish muscle amylase, as for amylases from other sources, activation is necessary. The boiled extracts have completely lost their amylytic activity. Parallel determinations of inorganic phosphate showed us that the splitting of starch is not affected by the enzymes of the phosphorylating systems.

TABLE 1
Amylytic Activity of Dialyzed Aqueous Extracts of Fish Muscle

Incubation time, min	Inactivated extract + NaCl		Without addition of NaCl		With added NaCl	
	color with iodine	reducing substances, mg/ml	color with iodine	reducing substances, mg/ml	color with iodine	reducing substances, mg/ml
			Experiment I			
0	Blue	0.00	Blue	0.00	Blue	0.00
30	Blue	0.00	Blue	0.00	Violet	0.059
90	Blue	0.00	Blue	—	None	0.129
150	Blue	0.00	Violet	0.023	None	0.184
			Experiment II			
0	Blue	0.00	Blue	0.00	Blue	0.00
60	Blue	0.00	Blue	0.00	None	0.067
120	Blue	0.00	Blue	0.012	None	0.156

The considerable activity of the amylase in the extracts which were studied indicates the presence of hydrolytic splitting of glycogen in fish muscles, but does not exclude, however, the possibility of a phosphorylative pathway for its splitting.

Some investigators have shown (on other subjects) that extracts from autolyzed muscle show much greater amylytic activity than extracts from nonautolyzed muscle. To test this with respect to fish, we carried out experiments with extracts of nonautolyzed muscle. They all showed insignificant amylytic activity. These results permit us to assume that during autolysis, amylase which is bound to protein is liberated so that the amylytic activity of aqueous extracts rises sharply, as was first shown by Bakh and Oparin [6] for plants and later also by other authors [1, 3, 5] for tissues of warm-blooded animals. According to the data of several authors, the amylytic activity of the digestive juices of fish vary sharply with the variety. Thus, Vonk [7] showed that the amount of amylase in the carp pancreas was 1000 times more than in pike pancreas. He related this difference to peculiarities in the diet of these fish. Similar differences are found in muscle amylases, but to a much smaller degree. Many of our experiments have shown that within the species, variations in amylytic activity of fish muscles do not exist.

Data exist [1, 3, 8] which indicate that the globulins which precipitate in the dialysis of the extracts, when they are added to the experimental mixture, increase the amylase activity, although they themselves evidently do not have amylytic activity. In special experiments we did not succeed in finding any particular activating effect of the globulins which precipitated during dialysis.

Optimum Conditions for the Action of Fish Muscle Amylase

NaCl concentration. Drozdov and Zhuravskaia [3] found that the optimal concentration of NaCl for activating cattle muscle amylase was 0.3%. It seemed of interest to establish the optimum NaCl concentration for the activation of fish muscle amylase. For this purpose we determined the activity of the amylase from the same extract with different NaCl concentrations.

The results (Table 2) show that the amylase of fish muscle extracts is most active at NaCl concentrations around 0.44-0.50%.

Reducing the NaCl concentration to 0.22% and increasing it to 0.66% or above hinders hydrolysis of starch by the muscle extracts. The optimum NaCl concentration for the amylase activation is evidently the same for different species of fish.

TABLE 2

Relation of Amylytic Activity of Fish Muscle Extracts to NaCl Concentration

Incubation time, min.	% concentration of NaCl							
	0.22		0.44		0.66		0.88	
	color with iodine	reducing substances	color with iodine	reducing substances	color with iodine	reducing substances	color with iodine	reducing substances
				Experiment I (shad)				
0	Blue	–	Blue	–	Blue	–	Blue	–
30	Blue	–	Violet	–	Blue-violet	–	Blue	–
90	Violet	0.084	None	0.147	None	0.116	Violet	0.073
				Experiment II (carp)				
0	Blue	–	Blue	–	Blue	–	Blue	–
90	Violet	0.093	None	0.136	None	0.098	Violet	0.067

Optimum temperature. According to the literature [1, 3, 7] the optimum temperature for most enzymes of skin and muscle of warm-blooded animals is 37-40°.

We would expect that the optimum temperature for fish muscle enzymes would be lower than for warm-blooded animals since fish usually live under colder conditions and their body temperature is always 2-3 times lower than the body temperature of warm-blooded animals. (The bodies of fish usually have the temperature of the surrounding medium).

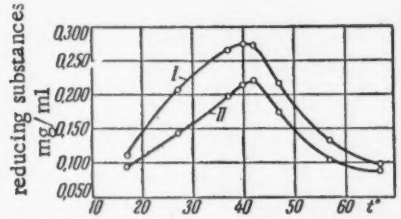

Fig. 1. Effect of temperature on amylytic activity of aqueous extracts of fish muscle. I) 2 hour incubation. II) 3 hour incubation.

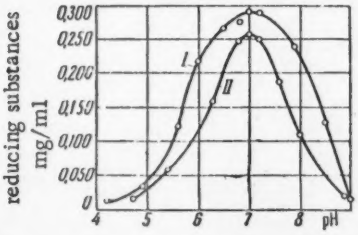

Fig. 2. Relation of amylytic activity of fish muscle extracts to pH of the medium. I) carp (Cyprinus carpio L.). II) shad (Trachurus trachurus L.) Incubation 3 hrs.

Our original assumption was not correct. We showed that fish muscle amylase, like that of warm blooded animals, was most active at 37-42°. The curves in Fig. 1 show the rise and fall in amylase activity with temperature. These results indicate that there is apparently no clear relation between the optimum temperature for fish muscle amylase and the temperature of the medium in which they live.

We should mention, however, that even at 27° the activity of fish muscle amylase is sufficiently high.

Effect of pH. There are almost no data in the literature on the optimum hydrogen ion concentration for the action of the different enzymes systems of fish. In order to determine the range of pH in which fish muscle amylase is most active, we measured this in fish muscle extracts in citrate and veronal buffer mixtures with different pH. The results of two of these experiments [for muscle extracts from carp (I) and shad (II)] are given in Fig. 2. The results show that the amylase of fish muscle extract is most active at pH 6.9-7.2. This optimum hydrogen ion concentration is the same for sea and fresh water fish. Nevertheless, for the sea fish the optimum pH for amylase activity (Fig. 2) varies within narrower limits than for the fresh water fish.

Activity of Fish Amylase in Muscles from Different Parts of the Body

We carried out comparative experiments to study the distribution of amylase in muscles from different parts of the body. As far as we could determine, such experiments have not been carried out for any fish enzyme, in spite of the importance of this question. As a result of our comparative experiments, we showed that the amylytic activity of the muscles from different parts of the body was not the same. As Fig. 3 shows, the tail muscles (motor muscles) have 1.5 to 2 times more amylytic activity than the rest of the skeletal muscles. Similar, but no so clearly shown differences are also found by comparing the amylytic activity of extracts from the body skeletal muscles with that of fin muscles. Thus, there is basis for the assumption that this difference in activity of the amylase is relatively great in the rapidly swimming fish (such as the trout) and less in the so-called "quiet" fish (as, for example, the carp).

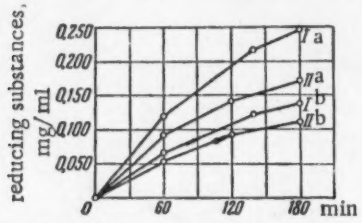

Fig. 3. Relation of amylytic activity of muscle extracts on the position of the muscles in the fish body. Ia) Extract from tail muscle of trout (Salmo trutta fario L.); Ib) the same from the skeletal muscle; IIa) Extract from tail muscle of carp (Cyprinus carpio L.); IIb) the same from skeletal muscle.

SUMMARY

As a result of investigations carried out on fish muscles we have shown considerable amylytic activity which is found in the muscles of both sea and fresh water fish. Fish muscle amylase is activated by chlorides (the optimum concentration of NaCl is 0.4 to 0.5%). Optimum action of fish amylase occurs at 37-42° and pH 6.9-7.2. From the nature of its action, fish muscle amylase belongs to the α-type. The amylytic activity of the muscles differs in different parts of the body.

LITERATURE CITED

[1] A. N. Petrova, Biokhimiia 11, 119 (1946).

[2] A. N. Petrova, Biokhimiia 12, 209 (1947).

[3] N. S. Drozdov and N. K. Zhuravskaia, Biokhimiia 21, 182 (1956).

[4] A. G. Kritskii, Biokhimiia 15, 334 (1950).

[5] J. B. Sumner and G. F. Somers Chemistry of Enzymes and Methods for Their Study [Russian translation] (IL, Moscow, 1948).

[6] A. N. Bakh, Collected Works of A. N. Bakh on Chemistry and Biochemistry (Izd. AN SSSR, Moscow, 1950), p. 597.*

[7] H. J. Vonk, Z. Physiol. 5, 453 (1927).

[8] M. D. Kiverin and M. A. Ritt, Biokhimiia 18, 257 (1953).

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THE EFFECT OF MITOCHONDRIA ON GLYCOLYSIS

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The development of techniques for the fractionation of intracellular tissue components by differential centrifugation has opened up new possibilities for the investigation of an outstanding interesting problem concerning the correlation between glycolysis and respiration within the cell. Numerous investigations showed that the glycolytic reactions were localized within the soluble fraction, that is, the fraction remaining after the sedimentation of the nuclei and cytoplasmic particles [1-2], while the oxidative systems were localized mainly in the mitochondria [3].

It was quite natural to assume that, when the interrelationship of the glycolytic and the oxidative systems was studied after their isolation by the various centrifugal techniques, it should be possible to confirm the observations made by Engel'gardt and Sakov [4] in 1943, in model systems containing isolated glycolytic enzymes, concerning the effect of various oxidizing agents on such systems. It was demonstrated that the enzyme phosphofructokinase, which transfers a phosphate group from ATP to fructose monophosphate, showed a high degree of sensitivity toward a variety of oxidizing agents.

The original work of Graffi and Schneider [5] showed that complicated interrelationships existed between the anaerobic glycolysis of the liver homogenate fraction, and the mitochondria added to it. In experiments where glycolysis was estimated by lactic acid formation, large amounts of mitochondria added (dilution 1 : 2,5), as well as very small amounts (1 : 120), inhibited glycolysis to a certain extent, while intermediate concentrations showed a highly stimulating effect. Mitochondria isolated from tumors, and added to the system in the same concentration range, showed only a stimulating effect. A similar effect of tumor mitochondria on the glycolysis in the soluble fraction was shown by Tiedemann et al [6, 7]. The authors explained the results on the basis of the regulating effect of the enzymes adenosinetriphosphatase [5] or hexokinase [7], which were found to be particularly active in tumor tissues.

The theory of Graffi et al. gained further support from their subsequent work [8] where, in the presence of versene as an ATP-ase inhibitor, the stimulating effect of the mitochondria was abolished completely.

Another group of authors [9, 10] showed that isolated liver mitochondria inhibited the aerobic glycolysis in soluble fractions prepared from brain and tumor homogenates; in these experiments the inhibition was demonstrated both by the fall in lactic acid production and by the decrease in substrate utilization. The authors claimed that this inhibition represented a type of Pasteur effect, namely, a depression of the activity of some glycolytic reaction by some enzymatic system of the mitochondria, most probably by the mitochondria-localized enzymes of the cytochrome chain.

On first examination the two sets of results obtained by the different authors were in complete disagreement: Graffi et al. observed mainly a stimulation of glycolysis by mitochondria, the system studied being anaerobic glycolysis, while Aisenberg et al. established an inhibition by mitochondria, glycolysis being determined from lactic acid formation under aerobic conditions. However, as established by the present authors, one deals here not with a different set of experimental conditions, but with a difference in the concentration of added mitochondria. This has also recently been borne out by the work of Schneider and Graffi [11].

The glycolytic system employed in the present work was the soluble fraction of a rat muscle homogenate in 0.25 M sucrose, obtained after sedimentation of all structural elements visible in the phase contrast microscope. Mitochondria were prepared in the usual way from rat liver homogenates and were washed twice with sucrose solution. Glycolysis was determined as lactic acid formation, the latter being estimated according to Barker and Summerson, with a number of modifications necessitated by the presence of high amounts of sucrose in the solutions. The values for lactic acid (LA) formation were expressed as μl lactic acid formed under standard conditions (usually in 40 min at 37°) per 100 mg of wet weight of tissue.

Substrates and cofactors were added in the following concentrations: ATP – 0.001 M; hexose diphosphate (HDP) – 0.005 M; glycogen – 0.1%, nicotinamide – 0.004 M; glucose-6-phosphate (G-6-P) – 0.005 M; $MgCl_2$ – 0.005 M; phosphate buffer, pH 7.3 – 0.015 M; TRIS buffer – 0.005 M.

Presented in Fig. 1 are the results of an experiment dealing with the effects of various concentrations of mitochondria on glycolysis in the soluble fraction. Glycogen served as a substrate. The mitochondrial concentrations have been expressed in terms of equivalent wet weight of liver. Experiments of this type were carried out simultaneously under aerobic conditions (under a mixture containing 5% CO_2 in oxygen) and anaerobically (under nitrogen).

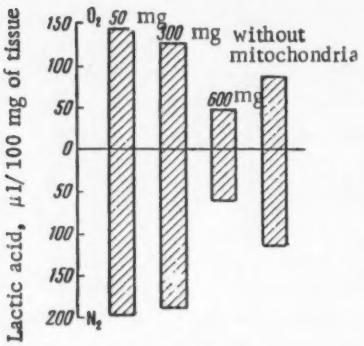

Fig. 1. The effect of various concentrations of mitochondria on the aerobic and anaerobic glycolysis in the soluble fraction. Concentration of mitochondria expressed in terms of mg of wet tissue.

It will be seen that the addition of mitochondria in amounts equivalent to 600 mg of wet liver already caused a considerable inhibition of glycolysis. When the amount of added mitochondria was increased to the equivalent of 800-1000 mg of wet liver, glycolysis was greatly, and in some cases, completely, inhibited. The addition of mitochondria in amounts equivalent to 300 mg of wet liver, or below this concentration, caused considerable stimulation of glycolysis.

The changes in glycolytic activity observed upon the addition of mitochondria were quite similar in each case under either aerobic or anaerobic conditions.

Thus, mitochondria in low concentration stimulated, and in high concentrations inhibited, the glycolytic activity. The divergent results obtained by Graffi et al. and by Aisenberg et al. may therefore be interpreted on the basis of the different concentration of mitochondria employed by the two groups – about the equivalent of 40 mg tissue in the former case, and about 600 mg in the latter – and not by the fact that Graffi et al. employed anaerobic conditions, while the other group worked aerobically.

The effect of mitochondria on glycolysis is therefore determined by the concentration of the particles added and, as will be shown below, depends on the substrate employed in the glycolytic reaction, but it is not at all dependent on the experimental conditions (the presence or exclusion of oxygen).

Mitochondria prepared from muscle, when added to the soluble fraction, exerted only a stimulating effect on glycolysis. Even in the case when muscle mitochondria were added in amounts equivalent to 1000-4000 mg of tissue (Table 1), no inhibition of glycolysis could be demonstrated. It should, however, be remembered that, in terms of total mitochondrial fraction protein, the yield of mitochondria from muscle was considerably lower than that from an equal weight of liver.

Independent checks with both the liver and muscle mitochondria, in the absence of the soluble fraction, but in the presence of all the cofactors and substrate, showed that these particles alone were not capable of producing lactic acid. Minute amounts of lactic acid were formed by liver mitochondria when the substrate employed was HDP; however, these amounts were too small to warrant attention.

Incubation of mitochondria in the presence of lactic acid also showed that this compound was not oxidized by mitochondria. Thus, the inhibition or stimulation of the glycolysis by mitochondria could not be explained by a possible production, or destruction, of lactic acid by the particles themselves.

In order to discover the possible sites of the stimulating and inhibitory effect of mitochondria in the glycolytic process, experiments were designed in which the effect of mitochondria was tested in the presence

of various individual substrates: glycogen, G-6-P and HOP. The results of these experiments have been presented in Table 2.

TABLE 1

The Effect of Muscle Mitochondria on Lactic Acid Formation (mitochondrial concentrations expressed in terms of mg of wet weight of tissue)

Mitochondria	LA formation, in μl/100 mg of tissue	
	in O_2	in N_2
Without mitochondria	15	17
500	25	26
1000	32	31
4000	31	35

TABLE 2

The Effect of Liver Mitochondria on the Glycolysis of Various Substrates
(LA formation in μl/100 mg of tissue)

Substrate	Expt No	expt'l cond.	without mitochondria	With mitochondria (equiv 800-1000 mg of tissue)
glycogen	32	в O_2	88	34
	32	» N_2	156	42
	33	» O_2	97	20
	33	» N_2	122	0
	38	» O_2	97	20
G-6-P	30	» »	112	42
	30	» N_2	126	75
	32	» O_2	104	41
	32	» N_2	153	32
	34	» O_2	131	14
	34	» N_2	71	24
HDP	30	» O_2	117	175
	30	» N_2	126	223
	35	» O_2	148	197
	38	» »	156	153
	41	» »	170	200
	42	» »	116	129

First, these results have confirmed that the choice of aerobic or anaerobic conditions had no effect on the outcome of the experiment. In experiments with three different substrates the effect of mitochondria on glycolysis was the same in each case in the presence or absence of oxygen. In order to establish this fact beyond any doubt, meticulous care was taken in some experiments to remove all traces of oxygen, since it could be argued that the inhibitory effect of mitochondria under anaerobic conditions may have been due to the presence of very small traces of oxygen, nevertheless sufficient to support a certain degree of aerobiosis. However, preliminary saturation of the experimental vessels with nitrogen, as well as the removal of all traces of oxygen from the nitrogen employed, did not affect the results.

It may also be seen from Table 2 that, with the large amounts of mitochondria employed (equivalent to 800-1000 mg tissue), the inhibitory effect on glycolysis with G-6-P as a substrate was the same as when the substrate employed was glycogen. The inhibition of glycogen glycolysis by mitochondria in the various experiments ranged from 60 to 100%, and in the case of G-6-P, 40-90%. It may therefore be concluded that the site of inhibition was somewhere beyond the G-6-P stage.

In the experiments with HDP as a substrate, the mitochondria added in high concentrations were found to exert a stimulatory, rather than inhibitory effect. Admittedly, the degree of stimulation was not very significant, and in some cases no effect on the glycolytic reactions beyond the HDP stage could be detected. It may nevertheless be stated that the inhibitory effect of mitochondria on glycolysis is localized somewhere between the G-6-P and HDP stages, in all probability at the stage previously investigated by Engel'gardt and Sakov, namely during the secondary phosphorylation of the ketose; the site of the mitochondrial inhibition is thus apparently the enzyme phosphofructokinase.

The stimulating effect of mitochondria is apparently sited at some stage of glycolysis beyond HDP. Furthermore, in the presence of HDP as a substrate, glycolysis was stimulated by both high and low concentrations of added mitochondria.

The fact that very slight stimulation, or sometimes its complete absence, was observed in the experiments described above may have been due to a possible glycogen resynthesis during the prolonged incubation period; the appearance of the latter substrate would set in motion other glycolytic reactions, in which the high concentrations of mitochondria are inhibitory.

This hypothesis was tested experimentally by observing the effect of high concentrations of mitochondria on glycolysis over varying intervals of time, using HDP as a substrate (Fig. 2).

It was actually found that, in the short incubation period (10-20 min), the stimulating effect of mitochondria on lactic acid formation from HDP was very clearly demonstrated. This stimulating effect was gradually levelled off as the incubation time was increased (30-40 min).

The results thus give the impression that the inhibitory effect of mitochondria on glycolysis takes place at some stage prior to HDP, while the stimulation occurs somewhere beyond that stage; also, in the case of high mitochondrial concentrations, the inhibitory effect is considerably stronger than the stimulating one, which may only be demonstrated in the absence of the pre-HDP glycolytic steps, and in the presence of low mitochondrial concentrations, which would not be sufficient to establish a masking inhibitory effect.

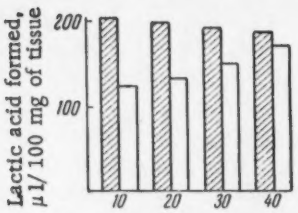

Fig. 2. The importance of the incubation time in the demonstration of the stimulating effect of mitochondria on glycolysis in the presence of HDP as a substrate. Shaded columns) incubations with added mitochondria; plain columns) without mitochondria.

The theory about the existence of distinct sites for the inhibitory and stimulating effect of mitochondria suggests the possibility for separating these factors. Preliminary attempts at such a separation involved the use of filtrates obtained from mitochondrial suspensions after short term heat treatment (boiling). The filtrates were found to exert neither stimulatory nor inhibitory effect on glycolysis. The active components are therefore either thermolabile, or else failed to pass into the filtrate by virtue of being firmly bound to the precipitated protein.

Since the mitochondria represent a specific center of the cellular oxidative metabolism, and contain the mechanism of oxidative phosphorylation, it may be argued that the formation of the factors affecting glycolysis is somehow related to the specific mitochondrial reactions. However, when the mitochondria were incubated under conditions favoring oxidative phosphorylation, and the suspension centrifuged at 15,000 g, the supernatant fluid obtained failed to stimulate or inhibit glycolysis in the soluble fraction.

SUMMARY

Liver mitochondria in high concentrations were found to inhibit glycolysis in the soluble fraction from rat muscle, while in the same system mitochondria in low concentrations had a stimulating effect.

The effect of mitochondria on glycolysis was independent of the presence of oxygen. The inhibitory effect occurs at a stage between glucose-6-phosphate and hexose diphosphate while the stimulating effect is sited somewhere beyond the hexose diphosphate stage.

Muscle mitochondria exert only a stimulating effect on glycolysis.

LITERATURE CITED

[1] G. A. LePage and W. C. Schneider, J. Biol. Chem. 176, 1021 (1948).

[2] Chr. de Duve, H. C. Hers, and I. Berthet, Ind. Chim. belge 17, 143 (1952).

[3] G. H. Hogeboom, W. C. Schneider, and M. J. Striebich, Cancer Res. 13, 617 (1953).

[4] V. A. Engel'gardt and N. E. Sakov, Biokhimiia 8, 9 (1943).

[5] A. Graffi and E. J. Schneider, Naturwissenschaften 43, 376 (1956).

[6] H. Tiedemann and I. Born, Biochim. biophys. acta 27, 656 (1958).

[7] H. Tiedemann and I. Born, Z. Naturforsch. 13b, 268 (1958).

[8] A. Graffi, E. J. Schneider, G. Sydow, and L. Venker, Naturwissenschaften 44, 66 (1957).

[9] A. C. Aisenberg, B. Reinafarje, and V. R. Potter, J. Biol. Chem. 224, 1099 (1957).

[10] A. C. Aisenberg and V. R. Potter, J. Biol. Chem. 224, 1115 (1957).

[11] E. J. Schneider, A. Graffi, H. Bielka, and L. Venker, Naturwissenschaften 44, 446 (1957).

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ACTIVITY OF THE ENZYMES OF THE DI- AND TRICARBOXYLIC ACID CYCLE AS A FUNCTION OF THE PROTOPLAST STRUCTURE OF MICROCOCCUS LYSODEIKTICUS

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For some time past, investigations on the relationship between enzymatic processes and protoplasmic structure have proceeded mainly in the direction of isolation of various structural elements of cells and determination of individual enzymes or complicated enzyme systems in them. Such experiments were carried out with mitochondria, microsomes, nuclei and plastids of animal and vegetable cells, and granules obtained from bacterial cells. However, the study of enzyme systems in structural elements isolated from cells cannot give a complete picture of the role of protoplasm as a whole in integrating the various complicated enzymatic processes taking place in cells. From this point of view, investigations on protoplasts obtained by the dissolution of the bacterial cell membranes under defined osmotic conditions, are very desirable. Protoplasts prepared under such conditions represent structural fragments of protoplasm, more or less distinguishable from the parent intact cells. By varying the osmotic pressure of the medium surrounding the cells, it is possible to obtain protoplasts of different structure, and consequently of different biochemical characteristics.

It was demonstrated in our previous papers that the respiratory processes and the incorporation of labelled amino acids in the protoplasts of Micrococcus lysodeikticus are inhibited by reducing the saccharose content of the medium below a definite concentration [1, 2].

The mechanism of inhibition of the above processes, apparently, lies in the derangement of the protoplasmic structure as manifested in the spatial uncoupling of enzymes, coenzymes, and substrates and also in the modification and gradual disappearance of the extensive surface of the microheterogeneous phases of the protoplasm on which the various enzymatic reactions take place in the cell. The various links of the complicated enzymatic processes are, probably, damaged to varying degrees during the gradual derangement of the cellular structure. The problem of the differential damage to the respiratory enzymes during gradual derangement of the protoplasmic structure was studied during the present work. The data obtained might further the understanding of the spatial organization of the enzymatic processes in the protoplasm.

METHODS

Cultures of the bacteria Micrococcus lysodeikticus, Flemming's strain, were used in these experiments. The culturing of the bacteria and preparation of the bacterial mass were carried out as described earlier [1]. Lysozyme was prepared by the method of Alderton et al. [3]. Lysis was carried out in phosphate buffers (pH 7.0) of the following concentrations 0.5, 0.4, 0.3, and 0.2 M. One ml of the phosphate buffer contained 1.5 mg of NaCl. The concentration of the bacterial mass was held constant and consisted of 50 mg per ml (moisture 75%). To this mixture was added 0.05 ml of phosphate buffer containing 200 μg of lysozyme and 25 μg of $MgSO_4$. Lysis was carried out for 30 minutes at 37°.

Oxygen absorption was determined by Warburg's method at 30°. Substrates for oxidation were 0.5 M solution of the sodium salt of succinic acid and 1 M solutions of the sodium salts of acetic, pyruvic, α-

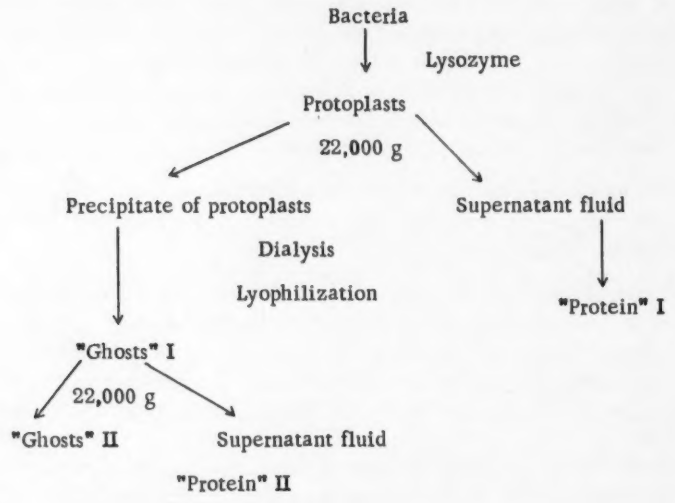
ketoglutaric, L-malic and citric acids. In these experiments, lysis was carried out in Warburg vessels containing 1 ml of cell suspension and lysozyme, as described above. After termination of lysis, 0.1 ml of neutral solution of the corresponding substrate was added. Nitrogen was determined by Kjeldahl's method. Spectrophotometric measurements were carried out in a SF-4 spectrophotometer. Specimens for electron microscopy were prepared by the method described earlier [1].

The activity of succinic oxidase was determined according to Singer and Kearney [4]. To 0.5 ml of phosphate buffer containing 3 mg of dry preparation were added 0.1 ml of 0.5 M sodium succinate, 0.1 ml of 0.3 M KCN and 1 ml of a solution of 2,6-dichlorophenolindophenol in 0.1 M phosphate buffer, pH 7.0. The dye solution was prepared in such a manner that the initial extinction of the sample was 0.800 at 600 mμ. The reaction proceeded for 5 minutes at 30°, after which the enzyme was inactivated by the addition of 3 ml of ethyl alcohol. The proteins were removed by centrifugation at 6000 rpm, and the extinction was measured at 600 mμ, the absorption maximum of the color. The reduction in color of a sample to which succinic acid was not added was measured simultaneously. Succinic oxidase activity, ΔE, is the difference between the extinctions of the sample and the control containing 3 mg of the dry preparation. Measurements were made against a solution containing all the components except the dye and the preparation. Nonenzymatic reduction of color by boiled preparations was not observed.

Preparation of Protoplasts and Cytoplasmic Membranes, "Ghosts"

To study the dependence of the enzymatic reactions upon the structure of protoplasts, the latter were prepared in solutions of various phosphate concentrations, following which the cytoplasmic membranes, called "ghosts", were obtained from the protoplasts. The experiments were carried out according to the following scheme.

Scheme for the preparation of Protoplasts and "ghosts" of M. lysodeikticus



As is apparent from the electron micrographs shown in Fig. 1, the protoplasts were completely free from admixture with intact cells. In structure, the protoplasts prepared in 0.5 and 0.4 M phosphate bore the greatest similarity to intact cells. There was a significant loss of density and clarity of outline of protoplasts from 0.3 M phosphate. These phenomena were particularly noticeable in protoplasts prepared by lysis in 0.2 M phosphate. For the determination of protein nitrogen in protoplasts, they were separated from the surrounding solution by centrifugation at 22,000 g for 15 minutes in a MSE centrifuge at 4°. The quantity of bacteria used in these experiments varied from 1500 mg in the case of protoplasts from 0.2 M phosphate to 2500 mg in the case of protoplasts from 0.5 M phosphate (moisture 75%). Two aliquots were taken from the supernatant fluid and mixed with equal volumes of 0.25% uranium nitrate in 5% TCA to precipitate the proteins and nucleic acids. The precipitate was separated by centrifugation for 30 minutes, and transferred quantitatively to a Kjeldahl flask for the determination of nitrogen. The nitrogen content of protoplasts was calculated by the following equation:

Protoplast N = N in complete bacterial lysate − N in supernatant fluid

Data regarding protoplast nitrogen and the absorption in the ultraviolet of the supernatant fluid immediately after the separation of the protoplasts by centrifugation are presented in Fig. 2. All the figures were calculated on the basis of 1.25 mg of dry bacteria in the initial sample. 10% of the protein nitrogen of the bacteria goes into solution on lysis in 0.5 M phosphate, 16% in 0.4 M, 44% in 0.3 M, and 60% in 0.2 M. The quantity of the dissolved substances which have maximum absorption at 260 and 280 mμ also increases with the decrease in phosphate concentration of the surrounding medium. These data indicate that a definite part of the cellular content passes into the medium on lowering the phosphate concentration of the medium.

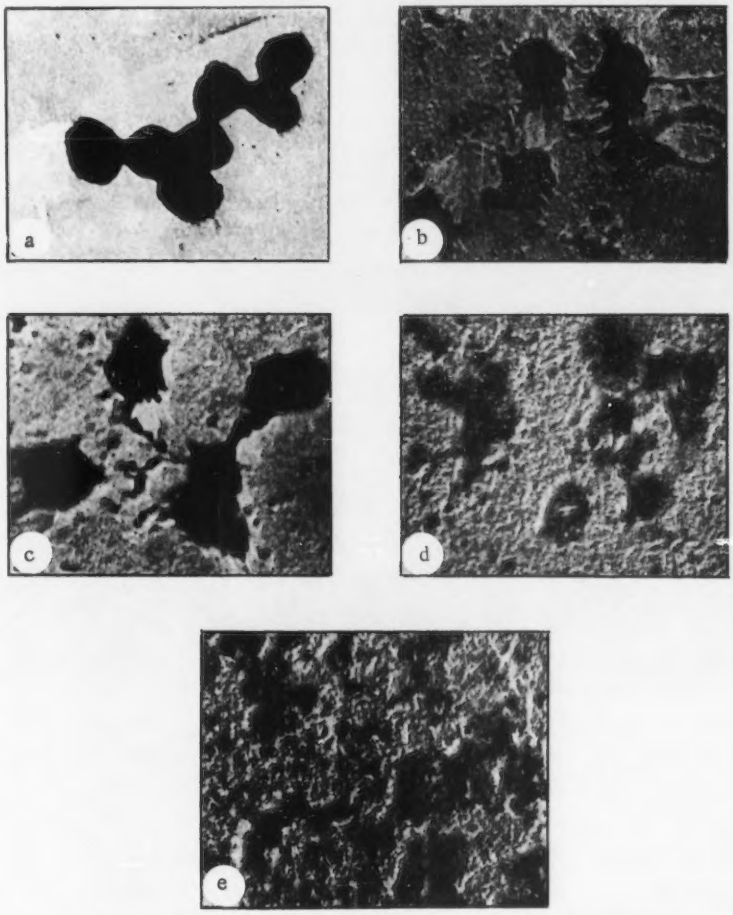


Fig. 1. Cells and protoplasts (14,000 x). a) Cells; b) protoplasts from 0.5 M phosphate; c) protoplasts from 0.4 M phosphate; d) protoplasts from 0.3 M phosphate; e) protoplasts from 0.2 M phosphate.

The precipitates of protoplasts obtained after centrifugation at 22,000 g had a yellowish tinge and did not dissolve in water, in contrast to those prepared in the presence of saccharose. The former were suspended in water (20 ml) and carefully stirred in the cold for 5 minutes; the suspension so obtained was dialyzed for 70 hours at 2° and dried by lyophilization. The preparations obtained by this procedure have been designated "ghosts" I.

The preparations designated "protein" I were obtained from the supernatant fluid after dialysis and drying. Nitrogen content of the fraction "ghosts" I was 11.8-12% of dry weight, and of the fraction "protein" I, 4.7-5.0%.

The photographs of "ghosts" I obtained by the above procedure from protoplasts prepared in 0.5, 0.4, and 0.3 M phosphate are presented in Fig. 3. These structural fragments of protoplasm ("ghosts" I) resemble, in their external appearance, the cytoplasmic membranes investing the bacterial cells, described first by Weibull [5], who called them "ghosts". Preparations from protoplasts obtained in the presence of 0.3 M phosphate contain comparatively few undamaged "ghosts" and a large number of compact fragments which probably, were produced by the treatment of protoplasts with 0.3 M phosphate. According to the data of Weibull [6], the yellow cellular pigment of M. lysodeikticus is concentrated in "ghosts". Consequently, the presence of pigment can be used as a criterion of the existence of definite structural fragments of protoplasm in our preparations.

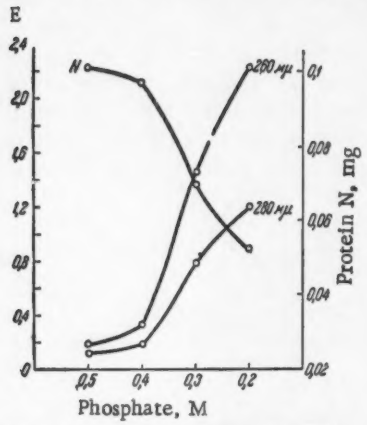


Fig. 2. Protein N of protoplasts from 0.5, 0.4, 0.3, and 0.2 M phosphate and the absorption of the supernatant fluid at 260 and 280 mμ.

The absorption spectra of "ghost" and "protein" fractions in the visible region, are depicted in Fig. 4. The pigment was extracted by boiling 20 mg of the preparation in 96% alcohol for 5 minutes. It is evident from the spectra presented that the pigment is connected with the structural elements — "ghosts" or their fragments. It is also obvious that these elements were absent in the supernatant fluid. The pigment was strongly bound in the "ghosts" and could not be removed by dialysis.

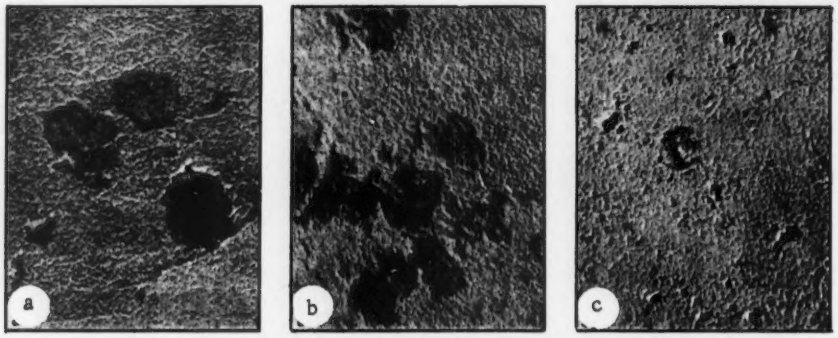


Fig. 3. Cytoplasmic membranes, "ghosts" I (14,000 x). a) "Ghosts" I from protoplasts in 0.5 M phosphate; b) "Ghosts" I from protoplasts in 0.4 M phosphate; c) "Ghosts" I from protoplasts in 0.3 M phosphate.

"Ghost" I preparations were subjected to further fractionation. For this purpose, 60 mg of the preparation was suspended in 20 ml of 0.1 M phosphate and centrifuged at 22,000 g. The precipitate of "ghosts" II contained the pigment, as is apparent from Fig. 4. On centrifugation of the suspension of "ghost" II fraction, soluble proteins constituting 50% of the total protein mass of the initial "ghost" I fraction, passed into the supernatant fluid.

The following preparations were thus obtained by the gradual destruction of the structure of bacterial cell: 1) protoplasts from 0.5, 0.4, 0.3, and 0.2 M phosphate; 2) "ghosts" I, prepared from protoplasts; 3) "ghosts" II, prepared from "ghosts" I.

Activity of the Enzymes of the Di- and Tricarboxylic Acid Cycle in Protoplasts

The activity of several enzymes of the di- and tricarboxylic acid cycle was determined in the protoplasts of different structure, by measuring the oxygen absorption

$$Q_{O_2} = \mu l\ O_2/\text{mg protein N of protoplasts/30 minutes.}$$

The results were corrected for the endogenous respiration of protoplasts which was of a low order, Q_{O_2} = = 15 - 20.

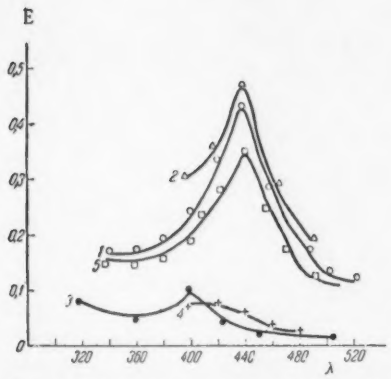


Fig. 4. Absorption spectra of alcoholic extracts of preparations "ghosts" I and II and "proteins" I and II. 1) "Ghosts" I; 2) "Ghosts" II; 3) "Protein" I; 4) "Protein" II; 5) complete bacterial lysate.

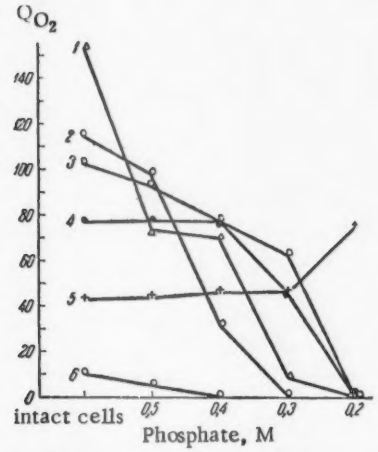


Fig. 5. Oxygen absorption by protoplasts and intact cells. The samples contained sodium salts of the following acids: 1) α-ketoglutaric; 2) pyruvic; 3) acetic; 4) succinic; 5) L-malic; 6) citric.

As is apparent from the data in Fig. 5, protoplasts oxidized pyruvic acid at the highest rate. This process was exceedingly sensitive to the derangement of structure of protoplasts. Pyruvic acid is not oxidized even by protoplasts from 0.3 M phosphate. An analogous picture is observed in the case of α-ketoglutaric acid which is feebly oxidized, by protoplasts prepared in 0.3 M phosphate. Apparently, the complicated enzyme system carrying out the oxidative decarboxylation of ketoacids requires for its activity a precise spatial organization of protoplasm. The nature of this system was not studied in our present investigations on bacteria.

Indirect evidence for the formation of active acetate during decarboxylation of pyruvic acid is the rapid utilization of acetic acid by protoplasts as judged from oxygen absorption (Fig. 5). It is known that the oxidation of acetic acid in the Krebs cycle must be preceded by its activation. The enzyme system of the protoplasts, which carries out the activation of acetic acid and the further conversion of the active acetyl, is comparitively stable because the oxygen absorption is completely reduced only when the protoplasts are prepared in 0.2 M phosphate. Utter, Krampitz and Werkman [7] have demonstrated that acetyl phosphate is utilized by protoplasts of M. lysodeikticus, prepared in the presence of 0.4 M NaCl.

In the light of the above-mentioned communication regarding the activation of acetic acid and the rapid oxidation of the resulting product, the slow rate of oxidation of citric acid observed by us, is surprising. A similar phenomenon was observed by Utter et al. [7] in their experiments with M. lysodeikticus. At the same time, Saz and Krampitz [8] discovered that the protoplasts of these bacteria incorporated labelled acetate and they detected the label in citric acid.

The oxidation of succinic acid, which is accomplished by a system of enzymes grouped under the general name, succinic oxidase, takes place at the same rate in protoplasts from 0.5 and 0.4 M phosphate; the rate of oxidation is somewhat reduced in protoplasts from 0.3 M phosphate but is completely blocked in protoplasts from 0.2 M phosphate. The relation of succinic oxidase to the derangement of protoplasmic structure is of particular interest in connection with the investigations of Tissières [9] and Stanier et al. [10] which have demonstrated that succinic oxidase is localized in the granules of the bacterial cell having a diameter of the order of 100-200 Å. At the same time, this enzyme system was detected only in "ghosts" [11]. According to the opinion of Mitchell and Moyle [12], "ghosts" consist of granules and that is why succinic oxidase and several other enzymes localized in "ghosts" can be detected in granules.

In our experiments, we could not detect the oxidation of succinic acid, as measured by oxygen absorption, by protoplasts from 0.2 M phosphate, which also contained structural fragments of protoplasm (Fig. 1, d). It is perfectly obvious that the spatial organization of the protoplasm has a great significance for the activity

of succinic oxidase and that a high level of organization, which is present in "ghosts", is essential for the process of transfer of electrons from succinic acid, across succinic dehydrogenase, a series of cytochromes and cytochrome oxidase,to oxygen.

A different picture is observed in the case of the system oxidizing malic acid, the activity of which is not appreciably altered by modification of the protoplasmic structure. The data cited, which are based on oxygen absorption per mg of protein nitrogen of protoplasts, reveal the high rate of oxidation of malic acid. This phenomenon is apparently explained by the oxidation of this substrate, not by the whole protoplast, but only by the cytoplasmic membrane of the "ghost", as will be shown below.

Oxidation of Malic and Succinic Acids by "Ghosts" I and II

After determination of the oxidation of a series of substrates, the activity of these processes was measured in structural fragments obtained by partial destruction of protoplasts, namely, in "ghosts" I and II.

Preparation of "ghosts" did not oxidize pyruvic or succinic acids. The addition of acetic acid was also not accompanied by a measurable oxygen absorption. The "ghost" preparations actively oxidized malic acid while their enzymes oxidizing the above substrates were completely inactivated. In experiments on the measurement of oxidation of malic and the above-mentioned acids, 0.05 ml of methylene blue (1 :1000) was added to the vessel. The oxygen absorption increased by 25-30 % in the presence of methylene blue. The malic acid-oxidizing enzyme system is localized in the cytoplasmic membranes ("ghosts") (Table 1).

TABLE 1
Oxidation of L-Malic Acid by Preparations of "Ghosts" I and II

Phosphate, M	Q_{O_2}	
	"Ghosts" I	"Ghosts" II
0.5	76	82
0.4	76	80
0.3	–	80
0.2	62	80

It can be concluded from the data cited, that the activity of the malic acid-oxidizing enzyme system is of a similar magnitude in the various "ghost" preparations obtained from protoplasts which differ greatly in their structure, but, apparently, contain cytoplasmic membranes or their fragments that can be precipitated at 22,000 g. The oxidation of malic acid is accompanied by energetic evolution of CO_2 (respiratory quotient 1.16). It was observed in later experiments that "ghosts" I contain an active β-decarboxylase which splits CO_2 from oxaloacetic acid. On the basis of joint localization of malic acid oxidizing enzyme system and β-decarboxylase, one can postulate the existence of "malic enzyme" system in the cytoplasmic membranes ("ghosts") obtained from the bacteria M. lysodeikticus [13]. This question was not elucidated in the present investigation. However, we must mention that the oxidation of malic acid by intact cells as well as protoplasts and "ghosts" was not depressed in our experiments by 0.01 M KCN independently from the presence of methylene blue. This shows that the terminal system participating in the oxidation of malic acid is a flavoprotein and not cytochrome oxidase. The presence of one of the enzymes, namely, $DPNH_2$-oxidase in granules of A. vinelandii has been shown by Alexander and Wilson [14].

Disruption of "ghosts" I by treatment of the precipitate of protoplasts after centrifugation with desoxyribonuclease and water, followed by dialysis and lyophilization, blocked the process of oxidation of malic acid ($Q_{O_2} = 18$). Malic acid was not oxidized by fractions "protein" I and II before or after dialysis. Mitchell and Moyle [12] observed in their experiments that the entire activity of the malic acid oxidizing enzyme system of Staphylococcus aureus resided in the "ghosts". At the same time, Alexander and Wilson as well as Stanier et al. reached the conclusion that the entire activity of this enzyme resides exclusively in the granules of A. vinelandii and P. fluorescens. One should point out that "ghosts" could not be preserved in the experiments of these authors. The work of Cota-Robles, Marr and Nilson [15] published recently, has established that the granules of Azotobacter agile contain the entire malic oxidase, succinic oxidase and several other enzymes and that these granules appear on the disruption of the cell membrane.

These authors could not separate the cytoplasmic membranes of bacteria and suggested that the above-mentioned enzymes are localized in some layers of the protoplasm densely adherent to the membrane.

Dialysis, washing and centrifugation carried out for obtaining the preparations of "ghosts" had no effect on the activity of malic acid-oxidizing enzymes. According to the data in the literature, the initial dehydrogenation of malic acid might be accomplished by dehydrogenases containing either diphosphopyridine nucleotide or triphosphopyridine nucleotide as a coenzyme. It is known that the coenzymes of dehydrogenation are easily removed by dialysis. Our experiments confirm the conclusion of Alexander [16] that one of the peculiarities of bacterial protoplasm is the strong bond between submolecular coenzymes and the structural elements.

The above-mentioned experiments on oxygen absorption by protoplasts during the oxidation of succinic acid showed that this process was blocked by derangement of protoplast structure. It can be postulated on the basis of these data that the terminal part of the enzymatic chain of succinic oxidase, namely cytochrome oxidase, was inhibited under the conditions of our experiments.

To clarify the localization of the enzyme complex of succinic oxidase, we tried to determine its activity by the reduction of a synthetic hydrogen acceptor by "ghosts" instead of oxygen absorption. 2,6-dichlorophenolindophenol was used as an acceptor. The site of entrance of this compound in the enzymatic chain of succinic oxidase was not elucidated; however, according to the data of Singer and Kearney, it is one of the important synthetic acceptors of hydrogen, and with its use one can determine the activity of succinic oxidase below the level of cytochrome oxidase. As might have been expected, the activity of cytochrome oxidase could not be detected in "ghosts" since the rate of color reduction in the presence of 0.015 M potassium cyanide was the same as in its absence. As measured by oxygen absorption, cyanide suppresses succinic oxidase activity of intact cells by 90%. Results of the measurement of the activity of succinic oxidase complex by the reduction of 2,6-dichlorophenolindophenol in consecutive preparations of "ghosts" are cited in Table 2.

TABLE 2

Reduction of 2,6-Dichlorophenolindophenol by "Ghost" I and II Preparations

Phosphate, M	ΔE	
	"Ghosts" I	"Ghosts" II
0.5	0.080	0.100
0.4	0.080	0.090
0.3	0.060	0.090
0.2	0.105	0.100

It is apparent from the data cited that activity of a component of the succinic oxidase complex, as measured by 2,6-dichlorophenolindophenol resides in the cytoplasmic membranes – "ghosts". This component is very labile and is easily destroyed. Thus, the disruption of "ghosts" as a result of the treatment of the protoplast precipitate with desoxyribonuclease immediately after centrifugation, leads to the suppression of activity by 75-80%, as measured by the oxidation of malic acid.

It is interesting that the activity of enzymatic reduction of 2,6-dichlorophenolindophenol is of a similar magnitude in "ghost" preparations obtained from protoplasts of different structure. Apparently, succinic oxidase as well as the malic acid-oxidizing system are localized in "ghosts" but the terminal part is destroyed even in protoplasts. The reduction of 2,6-dichlorophenolindophenol, which determines only a part of succinic oxidase, also depends upon the structural state. Thus, the rate of color reduction by intact cells, dried by lyophilization, exceeds that of "ghosts" by 50 times. Thus, the presence of a complicated structure of the type seen in protoplasts and resembling intact cells is essential for succinic oxidase activity.

On disruption of protoplasts, succinic oxidase activity is lost and only a part of enzymes remains bound to the cytoplasmic membranes – "ghosts", the disruption of which leads to the complete inhibition of the system.

SUMMARY

The oxidation of substrates by enzymes of the di- and tricarboxylic acid cycle depend on the structural state of protoplasm. The processes of oxidative decarboxylation of pyruvic and α-ketoglutaric acids are particularly sensitive to derangement of structure and can be accomplished only by protoplasts of definite structure and not by "ghosts".

Preservation of a definite level of protoplast structure is essential for the activation of acetic acid and its further oxidation.

Upon modification of the protoplast structure, succinic oxidase is at first inhibited because of the inhibition of the terminal enzyme system, cytochrome oxidase. A definite component of the succinic oxidase can

be detected in "ghosts" by the reduction of 2,6-dichlorophenolindophenol. On this basis, we agree with the opinion of several authors that succinic oxidase is localized in "ghosts".

In contrast to succinic oxidase, the malic acid-oxidizing system does not require a high level of structural organization for its activity. Malic acid is oxidized at a similar rate by protoplasts and their "ghosts" I and II. The greater stability of the malic acid-oxidizing system is probably due to the fact that it does not involve cytochrome oxidase, the oxidation being accomplished by flavoproteins. The entire malic acid-oxidizing enzyme system is concentrated in the "ghost" fraction.

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LITERATURE CITED

[1] A. I. Oparin, N. C. Gel'man, and I. G. Zhukova, Doklady Akad. Nauk SSSR 105, 1036 (1955).

[2] A. I. Oparin, N. C. Gel'man, and I. G. Zhukova, Biokhimiia 22, 399 (1957).*

[3] G. Alderton, W. Ward, and H. Fevold, J. Biol. Chem. 157, 43 (1945).

[4] T. P. Singer and E. B. Kearney, Methods of Biochemical Analysis, 4, 307 (1957) (Intersc. Publ. New York).

[5] C. Weibull, J. Bacteriol. 66, 688 (1953).

[6] C. Weibull, J. Bacteriol 66, 696 (1953).

[7] M. Utter, L. Krampitz, and C. Werkman, Arch. Biochem. 9, 285 (1946).

[8] H. Saz and L. Krampitz, J. Bacteriol. 67, 409 (1954).

[9] A. Tissières, Nature 174, 183 (1954).

[10] R. Stanier, L. Gunsalus, and C. Gunsalus, J. Bacteriol. 66, 543 (1953).

[11] R. Storck and J. Wachsman, J. Bacteriol. 72, 784 (1957).

[12] P. Mitchell and J. Moyle, Biochem. J. 64, 19P (1956).

[13] H. Kornberg and N. Madsen, Biochem. J. 68, 549 (1958).

[14] M. Alexander and P. Wilson, J. Bacteriol. 71, 252 (1956).

[15] E. Cota-Robles, A. Marr, and E. Nilson, J. Bacteriol. 75, 243 (1958).

[16] M. Alexander, Bacteriol. Rev. 20, 67 (1956).

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* Original Russian pagination. See C. B. Translation.

DETERMINATION OF FREE FORMALDEHYDE IN THE PRESENCE OF ITS COMPOUNDS WITH AMINO ACIDS AND PROTEINS

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In view of the wide and ever-increasing practical applications of formaldehyde-protein reactions (preparation of vaccines and toxoids, production of certain synthetic resins, formaldehyde tanning in the leather industry, etc.) the study of such reactions has grown considerably during the past few years. A number of authors have drawn attention to the difficulties encountered in evaluating the results of analyses of formaldehyde reversibly combined with proteins and amino acids [1]; these difficulties are a considerable hindrance to the study of reactions between formaldehyde and proteins, and they result from the lack of an accurate method for the determination of unreacted, so-called free, formaldehyde.

The estimation of free formaldehyde is of even greater practical importance in connection with the preparation and use of vaccines and toxoids. The determination of free formaldehyde permits of the evaluation of the amount of formaldehyde bound in the inactivation process (bound formaldehyde is calculated as being the difference between total and free formaldehyde), it gives directly the amount of unreacted formaldehyde, the presence of which is undesirable in vaccines, since it gives harmful side effects, and, finally, it serves for the control of one of the most important steps in the process of manufacture of immunizing preparations, viz. the reaction between the agent undergoing inactivation and formaldehyde. This step is particularly critical in the case of manufacture of poliomyelitis vaccine. The methods used in industry for the determination of free formaldehyde do not give absolute, but only relative values, and they cannot be applied in research work on the preparation of polipmyelitis vaccine [2], because of the low concentrations of free and bound formaldehyde in such material.

The numerous published methods for determination of free formaldehyde in solutions all suffer from the disadvantage of changing its concentration, since they all involve dilution of the solutions, with considerable change in their pH, and the labile bond between formaldehyde and amino acids or a number of other compounds is particularly sensitive to such factors. From this point of view diffusion methods are to be preferred, but the existing ones for the determination of free formaldehyde are so imperfect [3, 4] that they also give only approximate answers.

We believe that the method described below gives trustworthy and sufficiently precise results.

We used a Conway microdiffusion unit for the analyses, with a modified inner chamber. The gas diffuses from the relatively large volume of solution under analyses, in the outer annulus, and is absorbed in the very small volume of water or buffer solution placed in the inner chamber, until equilibrium is reached between the formaldehyde concentrations of the fluids in the inner and outer chambers. As a result all three forms of formaldehyde making up the 'free formaldehyde' of the test solution are reconstituted in the inner chamber; these forms are monomeric unhydrated formaldehyde, monomeric hydrated formaldehyde (methyleneglycol), and hydrated polymer—polyoxymethyleneglycol. All these forms are so readily interconvertible that their existence does not constitute any hindrance to the establishment of an equilibrium between the two compartments of the Conway unit, at which the concentration of free formaldehyde in the outer compartment is reproduced in the inner one. All that is required in order to achieve this is that the conditions governing hydration and polymerization of formaldehyde should be the same in the test and the absorbent solutions. This involves identity

of pH, in the first place. During the process of diffusion there is no appreciable change in the formaldehyde content of the test solution, and there is hence no shift in the amino acid – formaldehyde equilibrium, since the amount of formaldehyde lost to the vapor phase and to the absorbent solution of the inner chamber is negligibly small.

In this way the concentration of free formaldehyde in the solution under analysis is reproduced in the absorbent solution, where its determination is not interfered with by the presence of reversibly bound formaldehyde.

METHODS

Apparatus (Fig. 1). The inner chamber of the Conway unit was modified in such a way as to bring the absorbent solution closer to the test solution, and to reduce the air space within the unit. The depth of the inner chamber of the modified unit was 1.5-2 mm, and its floor was situated close to the top of the inner well of the ordinary Conway unit. The diameters of the inner compartments of the units should not vary by more than 1 mm. The outside diameters of the units are not critical. Standard Conway units may also be used, but with these equilibrium is not reached for several days. All the results presented below were obtained using the modified units.

Procedure. The outer chamber of the unit is filled with the solution under analysis, being an aqueous solution containing free and bound formaldehyde, and water or buffer solution of the same pH as the test solution is placed in the inner chamber. The unit is filled to the levels as shown in Fig. 1, at which the conditions for diffusion of formaldehyde from the outer to the inner chamber are optimal. In filling the inner chamber we endeavor to have a very thin layer of liquid, with as high a surface to volume ratio as possible. Our modified units had an outside diameter of about 5.8 cm, and the diameter of the inner chamber was 2.7 cm, with the floor at a height of 1 cm. We introduce 20 ml of solution into the outer compartment, and 0.3 ml into the inner one. These amounts may be varied, according to the dimensions of the Conway units available in a given laboratory.

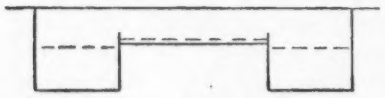

Fig. 1. Sketch of a Conway diffusion unit with a modified inner chamber. The broken lines represent the fluid levels in the filled unit. Cross section.

The filled, hermetically, closed units are left at room temperature. The formaldehyde concentration of the solution in the inner chamber is determined after 42-48 hours, by which time it will have become equal to that of the test solution. An exactly measured amount of solution is removed from the inner chamber, by means of a micropipette, or better still, an automatic micropipette. Any of the usual methods applied to determination of formaldehyde can be applied to the sample. The sensitivity and precision of the method proposed by us are limited by the sensitivity of the method applied to determination of formaldehyde in the absorbent solution. We used a calorimetric method (one of the most generally accepted and accurate ones) based on the color reaction given by formaldehyde with phenylhydrazine and potassium ferrocyanide in alkaline solution, as described by Tannenbaum and Bricker [5].

Since the formaldehyde concentration of the absorbent solution in the inner chamber is very close to that of equilibrium after 24 hours, it is possible, in case of need, to complete an analysis by the following day, but in that case an empirical correction, based on a control experiment, must be applied. The following example illustrates how this is done. After 28 hours of diffusion from a 0.004 M formaldehyde solution, containing 120.1 mg per liter, the concentration found in the inner chamber was 114.0 mg/liter, as compared with 102.0 mg/liter found for the unknown solution. The free formaldehyde content of the latter was thus $\frac{102.0 \times 120.1}{114.0} =$ $102.0 \times 1.053 = 107.4$ mg/liter.

Each analysis was carried out in duplicate.

Testing of the Method

The analytical conditions were worked out and tested on aqueous solutions of formaldehyde, prepared by dilution of formalin solutions. We first investigated the effects on the time required for equilibration of the concentrations of free formaldehyde between the test and the absorbent solutions, and on the extent to which

the concentrations in the outer and inner chambers were equalized, of varying the basic conditions (free formaldehyde concentration and pH of the solution under analysis) which determine the rates of polymerization, hydration, and diffusion of formaldehyde (all experiments were performed at room temperature, which was taken as being constant).

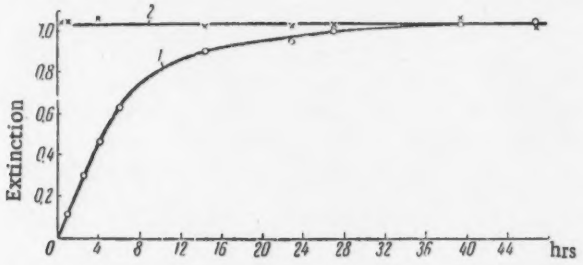

Fig. 2. Dynamics of diffusion of formaldehyde with time. Formaldehyde concentration – 0.003 M. 1) Optical densities found for the solutions in the inner chamber; 2) in the outer chamber.

The time required for establishment of equilibrium was derived from determinations of the formaldehyde concentration in both chambers of the units at various times. The results found for 0.03 M formaldehyde solutions are shown in Fig. 2.

The curve indicates that equilibrium is achieved after 42 hours of diffusion, but that the concentration of formaldehyde in the inner chamber is very close to the equilibrium value after 24 hours. There was virtually no change in the formaldehyde concentration of the outer chambers. Any variations found during the course of an experiment were within the range of experimental error of the colorimetric method used by us (± 2%).

A number of special control experiments showed that in all cases the concentration of formaldehyde had been equalized between the inner and the outer chambers within 42-48 hours. Leaving the systems for a further 24 hours did not affect this result.

We examined the effect of varying the formaldehyde concentration from 0.01% to 40%. The results of these experiments are shown in Table 1. In this Table, the concentrations of formaldehyde in the inner chambers are expressed as percentages of those in the outer chambers, for readier comparison, and in order to show how close the systems had approached to equilibrium.

As can be seen from Table 1, the relative amounts of formaldehyde absorbed in the inner chambers within 26 hours of diffusion were the same for all concentrations: the ratio $\frac{(F_{\text{inner chamber}})}{(F_{\text{outer chamber}})}$ was 94.1% ± 2.3. By 48 hr. 40 min. equilibrium had been attained in all cases. Thus, equilibrium was reached at the same time for all concentrations of formaldehyde. This finding is of special interest, in that dilution of formaldehyde solutions causes dissociation of the polymers, with increase in the partial pressure of formaldehyde vapor, calculated per mole of formaldehyde in solution [6]. The negative effect of this factor is manifested in Boyd and Logan's method for determination of formaldehyde [3]. Since the reverse effect applies to the process of absorption of the vapor, there is no net effect on the time required for establishment of equilibrium in our systems. It is clear from the data of Table 1 that our method is applicable over the whole range of formaldehyde concentrations.

Our subsequent experiments were performed on solutions of a low concentration of formaldehyde, such as are usually encountered in the biological products industry and in biochemical research.

The effect of pH was examined for formaldehyde concentrations of 0.12 and 0.16%, concordant results being obtained. The data of Table 2 apply to 0.16% solutions. A diffusion time of 42-48 hours was taken for these experiments.

As is evident from the table, the concentration of free formaldehyde present in all the test solutions, at different pH, was reproduced in the absorbent solutions of the same pH after 48 hours of diffusion, with an error not exceeding 2.5%. It is only necessary to adjust the pH of the absorbent solution when that of the test solution exceeds 10. At pH $\leq$ 10 the effect of pH may be ignored, and the method simplified by adding water to the inner chamber.

TABLE 1

Values of the Ratio $\frac{\text{(Formaldehyde in the inner chamber)} \times 100}{\text{(Formaldehyde in the outer chamber)}}$

After 26 Hours and 48 Hours 40 min of Diffusion With Various Concentrations of Formaldehyde in the Test Solutions

Formaldehyde content of solution under analysis (in outer chamber) g per 100 ml	Ratio of formaldehyde concentration in the inner chamber to that in outer chamber, as %	
	after 26 hrs of diffusion	after 48 hr 40 min of diffusion
0,01	93,3	102,1
0,08	96,0	100,2
0,16	91,8	101,7
0,80	92,3	99,5
8,0	95,2	98,3
20,0	95,6	97,0
40,0	94,4	100,0
Mean	94,1±2,3	99,8±2,8

Notes: The formaldehyde solutions were prepared by dilution of a formalin solution containing 40 vol % of formaldehyde.

The inner chambers were filled with water.

The systems were set up separately for each diffusion time.

In all cases the contents of the inner and outer chambers were analyzed for formaldehyde at the same time.

TABLE 2

Formaldehyde Content of the Solutions in the Inner Chamber After 48 Hours of Diffusion From Test Solutions of Different pH

(the solutions under analysis contained 0.16% formaldehyde)

Absorbent solution	Formaldehyde content of solution in inner chamber g per 100 ml				
	pH 1,9	pH 5,8	pH 7,0	pH 10,0	pH 11,6
Buffer solution of the same pH as the test solution	0,160	0,159	0,158	0,156	0,156
Water	0,161	0,158	0,159	0,158	0,149

Note: The buffer solutions were prepared in accordance with the directions of S. D. Balakhovskii and I. S. Balakhovskii [7]; pH 1.9— KCl-HCl; pH 5.8 and 7.0 — phosphate-borate buffer; pH 10.0-borax-NaOH; pH 11.6 — Na_2HPO_4— —NaOH.

No other sources of loss of formaldehyde during its estimation, such as, for example, Cannizzaro reactions, were found in our experiments.

One of the chief advantages of our method is, in our opinion, its applicability over a wide range of pH values and formaldehyde concentrations.

As regards the presence of inorganic salts, our results are in agreement with the findings of Boyd and Logan [3], that they do not effect the rate of diffusion of formaldehyde.

The results of parallel analyses may be summed up as shown in Table 3.

It is evident from Table 3 that the differences found between the results of parallel analyses were not greater than the experimental error of one of the most precise methods of determination of formaldehyde in the test solutions.

TABLE 3

Scatter of Results of Parallel Analyses (0.003 M aqueous formaldehyde solutions). The experimental results are expressed in terms of the extinctions found. A) Values found in samples taken from the inner chambers of 10 Conway units after a diffusion period of 45 hours; B) values found for samples of the initial solution

No. of expt	A	B
1	0,377	0,382
2	0,374	0,382
3	0,377	0,393
4	0,369	0,384
5	0,380	0,381
6	0,378	0,383
7	0,371	0,387
8	0,372	0,383
9	0,373	0,378
10	0,373	0,382
Mean	0,374	0,383
Amplitude of deviations	0,011	0,015
Mean deviation from the mean	0,0028	0,0025

Examples of analyses. Convenient material for testing our method of determining free formaldehyde is afforded by systems in which excess formaldehyde has been bound with bisulfite. Determination of free formaldehyde in the presence of the labile formaldehyde-bisulfite complex presents the same difficulties as with amino acid – formaldehyde complexes. For the testing of our method, a convenient aspect of complexing of formaldehyde with bisulfite is that it is known that these substrates react quantitatively in equimolecular proportions in aqueous solutions. Taylor and Moloney [4] were not able, in their experiments on the determination of free formaldehyde, to reproduce these proportions. The results obtained by us are presented in Table 4. The concentrations of total formaldehyde and bisulfite were determined by titration.

TABLE 4

Determination of Free Formaldehyde in the Presence of Its Complex with Bisulfite

(the concentrations are expressed as moles per liter of solution)

By titration method		Free formaldehyde, by the diffusion method
total formaldehyde	bound sodium bisulfite (bound formaldehyde)	
0,0043	0,0009	0,0035
0,0042	0,0019	0,0024
0,0043	0,0037	0,0006
0,0045	0,0039	0,0005

Note: Total formaldehyde was determined by the bisulfite method, as in Haiberger's quantitative method. Bound bisulfite was titrated with iodine after decomposing the bisulfite complex with formaldehyde by the action of weak alkali.

The molar concentration of bound bisulfite was equal to that of bound formaldehyde. The concentration of free formaldehyde was calculated as the difference between total and bound formaldehyde. The data of Table 4 permit of the comparison of the results given by the titration method with those given by our method. The results given by the two methods are practically identical, pointing to the trustworthiness of the values found using our method.

We present some examples of determination of free formaldehyde in the presence of formaldehyde-amino acid complex, using our method. These examples give an idea of the possibilities of the method for the study of reactions of amino acids with formaldehyde, at low concentrations.

Some of our results for determination of free formaldehyde in formaldehyde-inactivated virus suspensions, which are of considerable interest in the production of poliomyelitis vaccines, may serve as examples of the practical application of the method:

Batch 27 – 74.1 mg/liter (73.3% of total formaldehyde)
Batch 31 – 81.7 mg/liter (86.2% of total formaldehyde)

Batch 32 – 86.8 mg/liter (90.2% of total formaldehyde)
Batch 33 – 80.8 mg/liter (85.1% of total formaldehyde).

Total formaldehyde was determined in the distillate obtained by heating the solutions with dilute sulfuric acid.

TABLE 5
Examples of Determination of Free Formaldehydr in the Presence of Its Compounds Amino Acids (the initial concentrations of formaldehyde and amino acids were 0.01 M)

Amino acid	Molar concentration of free formaldehyde found		
	at pH 6,9	at pH 9.1	at pH 11,5
Glycine	0,0096	0,0082	0,0057
Serine	0,0095	0,0060	0,0048
Lysine	0,0095	0,0076	0,0020
Histidine	0,0008	—	—

Note: The monohydrochlorides of lysine and histidine were taken. The analyses were performed 24 hours after preparation of the solutions.

We were unable to find any free formaldehyde in 10 batches of poliomyelitis triple vaccine, to which sodium bisulfite had been added to about 0.004 M.

SUMMARY

A diffusion method is described, serving for the determination of free formaldehyde present in aqueous solutions together with formaldehyde reversibly bound to proteins, amino acids, and other compounds. The method is based on the principle of gas diffusion from a relatively large volume of solution under analysis to a very small volume of water or buffer solution, continued until the free formaldehyde concentration of the test solution is the same as in the absorbent solution.

The selectiveness of the method, which determines only free formaldehyde, together with its accuracy and simplicity, render it eminently suitable for both research and industrial purposes.

LITERATURE CITED

[1] H. Fraenkel-Conrat, B. A. Brandon, and H. S. Olcott, Biol. Chem. 168, 99 (1947).

[2] T. Wesslen, E. Lycke, S. Gard, and G. Olin, Arch. Ges. Virusforsch. 7, 125 (1957).

[3] J. Boyd and M. Logan, J. Biol. Chem. 160, 571 (1945).

[4] E. M. Taylor and P. J. Moloney, J. Amer. Pharmac. Assoc. Scient. Ed. 46, 299 (1957).

[5] M. Tannenbaum and C. Bricker, Anal. Chem. 23, 354 (1951).

[6] G. F. Woker, Formaldehyde Goskhimizdat, (Moscow, 1957).*

[7] S. D. Balakhovskii and I. S. Balakhovskii Methods for the Chemical Analysis of Blood (Medgiz, Moscow, 1953).**

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* Russian translation.
** In Russian.

SCIENCE NEWS

CONFERENCE ON THE BIOGEOCHEMICAL PROVINCES OF THE USSR

A conference dealing with the biogeochemical provinces of the USSR was held in Moscow, from November 20 to 24, 1957. It is called by the V. I. Vernadskii Institute of Geochemistry and Analytical Chemistry, AN SSSR.

The main objects of the conference were to review the results of research on biogeochemical provinces, to discuss the basic methods of study of such provinces and to coordinate further work on this subject. The conference also discussed a wide range of problems concerned with the migration of chemical elements in the soil and water, the part played by plants and animals in their transport, the concentration of elements by organisms, endemic diseases of plants, animals, and humans due to excess or deficiency of chemical elements in the environment, and the prevention of such diseases.

In view of the nature of the problems encountered in the study of biogeochemical provinces, the conference was attended by representatives of many different domains of the natural sciences, including biogeochemists, geochemists, pedologists, agricultural chemists, animal breeders, veterinarians, physicians, plant and animal biochemists, physiologists, geographers, geobotanists, toxicologists, hygienists, botanists, zoologists, biologists, microbiologists, biophysicists, histologists, and hydrobiologists. The conference was attended by 329 scientists from 42 cities of the Soviet Union, and also by physicians, biogeochemists, and biologists from the Chinese, Polish, and Rumanian Peoples'Republics. The proceedings included 34 papers read, with over 40 program discussions of them.

The participants stressed the methodological significance of the biogeochemical approach to the study of the processes of migration of chemical elements in different biogeochemical provinces. With such an approach, a given environment is regarded as a whole, and correlations are established between its chemistry (soil, natural waters, foods), its living organisms (plant and animal), their metabolic activities, and their morphological and chemical variability.

New data were presented in the papers and the discussions, relating to the trace elements (molybdenum, cobalt, copper, manganese, lead, zinc, boron, strontium, iodine, bromine, fluorine, etc.) of the soils, waters, plants and animal tissues of different zones of the USSR. In their consideration considerable importance was attached to the study of the compounds in which these elements occurred in nature. The processes of migration of chemical elements cannot be properly understood without a knowledge of the forms under which they existed under natural conditions. Of special interest is the study of compounds of these elements with products of degradation of organic substances in the soil (humic and fulvic acids, melanoidins, and glucosamine).

A number of speakers reported on their investigations of the metabolic mutability of organisms in different biogeochemical provinces, due to excess or deficiency of trace elements, in connection with the specific biochemical function of many of these elements. In such cases, metabolic variation may lead to morphological changes.

Certain important biological facts emerged from the study of biogeochemical provinces. Thus, a correlation was established between the cobalt concentration of the environment and synthesis of vitamin B_{12}, which contains cobalt, and the prevalence of acobaltosis and vitamin B_{12} deficiency (studies carried out in the biogeochemical provinces of the central nonchernozem zone which are deficient in cobalt, and in chernozem soil regions of normal and, in the Azerbaidzhan SSR, of excessive cobalt content). It was shown that deficiency of copper in the soil is the cause of reclamation disease and of nonmaturation of cereals (Polesie). Spring outbreaks of enzootic ataxy of sheep were shown to be related to the presence in the environment of copper and

lead in certain definite mutual proportions (in the biogeochemical province of the Tersk-Sulak- Kuma lowland). The distribution of endemic goiter was related to the presence of iodine deficiency in various parts of the USSR; the significance of cobalt deficiency in the distribution of enzootic thyroid enlargement of sheep was revealed (biogeochemical province of the central nonchernozem belt); a study was made of the ability of silt microorganisms of natural water reservoirs to synthesize vitamin B_{12} in cobalt-rich or deficient biogeochemical provinces; it was shown that an enzootic disease of animals characterized by corneal opacification and blindness was associated with considerable concentration of nickel in the corneal tissues (a nickel-rich biogeochemical province in the Aktiubinsk Oblast); variable and monstrous plant forms were also found in this province. A correlation was established between high strontium content of soils, waters, fodders and food products, and morbid conditions of the epiphyseal cartilaginous plate of animals and humans (the Urovsk endemic province of the Zeia River basin, in the Amur Oblast). It was shown that excessive intake of boron by animals interferes with kidney function, as a result of which boron accumulates in their organs and tissues, leading to outbreaks of enzootic enteritis (boron-rich biogeochemical province of North-Western Kazakhstan). Those plants of such provinces which concentrate boron were found to be morphologically variable. It was found that excess of molybdenum in soils, waters, and plants led to a heightening of xanthine oxidase activity of the tissues of animals living in such provinces, causing increased formation of uric acid, as a result of which endemic gout develops in the human population (molybdenum-rich province of the Ankavan area of Armenia). A study was made of certain biogeochemical provinces of the Samarkand and Altai Oblasts, and a study of the selenium-rich provinces of the Southern Urals and other parts of the USSR is in progress.

A declaration approved by the Plenary Session stated that the leading Soviet center for the investigation of biogeochemical problems and provinces is the V. I. Vernadskii Institute of Geochemistry and Analytical Chemistry, AN SSSR, which works in close contact with other research establishments of the Soviet Union.

The conference recommended the following measures for the further investigation of biogeochemical provinces: 1) to attract more workers from bordering specialities, such as geologists, geobotanists, soil scientists, chemists, physicians, agriculturalists, etc.; 2) considerably to widen the theoretical and practical scope of work on the geochemical ecology of wild and cultivated plants, and also of animals, by means of special experiments designed for the purpose; 3) to initiate the study of provinces in which there is an excess or deficiency of zinc, cadmium, manganese, mercury, chromium, antimony, selenium and sulfur, vanadium, magnesium, calcium, iron, sodium, and potassium; 4) to continue the holding of symposia on particular biogeochemical problems, at the Biogeochemistry Laboratory; 5) to assign the task of working out standard methods for the determination of elements in soils, waters, plants, and animal tissues, and to publish these methods in the form of a laboratory manual; 6) to arrange for the systematic publication of research papers from the Biogeochemistry Laboratory and from outside scientific establishments.

V. V. Koval'skii

CHRONICLE

TO THE MEMORY OF CLAUDE FROMAGEOT

(Obituary)

Professor Claude Fromageot, eminent French scientist, and Professor of Biochemistry of Paris University, died prematurely on January 10, 1956.

His name is familiar to biochemists of all countries, as well as to many scientists whose activities extended into the field of biochemistry. Claude Fromageot was born in Paris in 1899. He graduated from the French National Institute of Agriculture in 1922. He then began his scientific and teaching activities. Claude Fromageot devoted more than 36 years of his life to science and to the training of biologists, in particular of those specializing in various aspects of biochemistry. From 1929 to 1946, Claude Fromageot was a professor at Lyons University, and from 1946 to his death he was a professor of the University of Paris, where he was Head of the Department of Biochemistry from 1955.

At the onset of his scientific and teaching career Claude Fromageot worked in some of the leading biochemical laboratories of Europe and America – those of V. Henri, Warburg, Neuberg, and others. This led to his development as a biochemist of many-sided interests, whose researches covered a very wide field of biochemical problems.

To the end of his life, Claude Fromageot was a convinced advocate of international collaboration in the world of science. He was a member or an organizer of numerous international congresses, symposia, and the like, and was on the editorial board of a number of international journals.

Although Claude Fromageot never visited the USSR, he was well known to our scientists, and he had a high opinion of both classical Russian and Soviet biochemistry. Such was his interest in it that he learned to speak Russian, and to use it at meetings, for the study of Russian literature and other purposes.

The scientific activities of Claude Fromageot and his pupils covered a number of the cardinal problems of modern biochemistry: the amino acid composition and structure of proteins, oxidative processes in biological systems, the biosynthesis and degradation of amino acids, the intermediary metabolism of sulfur compounds, mechanisms of enzymatic catalysis, etc.

The work of Claude Fromageot and his school on these problems gave important results, which constituted a valuable contribution to world biochemistry. Fromageot's work on the structure of a number of protein macromolecules (such as insulin, lysozyme, vasopressin, and others) was of great interest. The results of his investigations into the mechanism of degradation of sulfur compounds were of particular importance. His researches on the desulfuration of cysteine and cystine by microorganisms prepared the way for the discovery of the reactions of desulfuration of cysteine, cystine, and homocysteine in animal tissues. Fromageot's laboratory then conducted a broad survey of the mechanisms of oxidative degradation of sulfur-containing substances of biological importance.

The work of Claude Fromageot's school was distinguished by the originality of its approach to problems, by its high standard of experimental performance, by the creative interpretation of the mechanisms of the processes investigated, and by the model way in which the experimental material was presented. It was in these ways that Claude Fromageot's eminence was best shown, and which enabled him to inspire numerous young workers, and to hand on to them the best traditions of French science.

Being opposed to anything that savored of routine in science, Claude Fromageot spared no effort to become familiar with new ideas and methods, thus, making an important contribution to the development of French biochemistry, in particular in the domains of enzymology and protein structure, which he considered to be inseparable aspects of modern biochemistry.

Not only was Claude Fromageot a talented organizer and research worker, but he was also a first-class teacher, able to arouse the keen interest of his audiences.

Soviet biochemists share the grief of their French colleagues in the loss suffered by French and world biochemistry with the death of that eminent scientist, Claude Fromageot.

M. A. Ter-Karapetian

ERRATA

Biochemistry Vol. 23, No. 5, 1958

Page	Line	Reads	Should Read
714	30	1.5-2 μ moles	$1.5\text{-}2 \cdot 10^{-3}$ μ moles

FOUNDING OF THE ALL-UNION SOCIETY OF BIOCHEMISTS

The All-Union Society of Biochemists was founded in accordance with a decision of the Praesidium of the Academy of Sciences USSR, of the 7th February, 1958.

The appointment of the following members of the Organizing Committee was approved, by the same decision:

A. I. Oparin (Chairman), A. Z. Babaev (AN Azerbaidzhan. SSR), A. N. Belozerskii, E. V. Budnitskaia (Responsible Secretary), V. N. Bukin, Kh. G. Bunatian (Arm. SSR), A. E. Braunshtein, G. E. Vladimirov (Biochemistry Section of the Society of Physiologists), T. I. Godnev (AN Beloruss. SSR), T. B. Darkanbaev (AN Kazakh. SSR), S. V. Durmishidze (AN Gruz. SSR), N. D. Ierusalimskii (Microbiological Society), A. L. Kursanov, V. N. Orekhovich, A. V. Palladini(AN Ukrain. SSR), S. E. Severin (Vice-Chairman), N. M. Sisakian, V. I. Tovarnitskii, R. V. Feniksova, M. M. Shemiakin (Mendeleev Society), A. A. Shmidt (AN Latv. SSR), V. A. Engel'gardt, S. Iu. Iunusov (AN Uzbek. SSR), V. V. Iurkevich (Sverdlovsk University), V. G. Iakovlev (AN Kirgiz. SSR).

The Organizing Committee was entrusted with the tasks of preparing the Charter of the Society, of preparing for the recruitment of members, and of arranging for the calling of an All-Union meeting of delegates.

The Organizing Committee assembled 4 times from March to August, 1958, to discuss the draft Charter of the All-Union Society of Biochemists, the program of work of the Organizing Committee, and plans for the recruitment of members of the Society in the Union Republics.

The establishment of local branches of the Society was discussed.

It was decided to call the meeting of delegates, which should elect the Central Council of the All-Union Society of Biochemists, at a time which would fit in with the Mendeleev Congress, from December 15-17, 1958.

The basic work on the organization of the Society of Biochemists in the Union Republics has now been completed. Councils of the Society of Biochemists have been elected, members have been recruited, and programs of work have been prepared in the Armenian SSR, the Azerbaidzhan SSR, the Belorussian SSR, the Kirgiz SSR, the Moldavian SSR, and also in Moscow, Leningrad, Gorkii, Sverdlovsk, and Rostov-on-Don.

The All-Union Society of Biochemists now has a membership of over 1500.

There are about 300 members of the Society of Biochemists in Moscow. S. E. Severin was elected Chairman of the Council of the Moscow Biochemical Society, with A. N. Belozerskii, A. E. Braunshtein, V. N. Bukin, and B. N. Stepanenko as Vice-Chairmen, and I. B. Zbarskii as Secretary.

The first meeting of the Moscow Society of Biochemists was held on October 2, 1958, at which N. M. Sisakian, A. E. Braunshtein, A. N. Belozerskii, V. L. Kretovich, V. N. Bukin, G. F. Gauze, and S. E. Severin reported on the IV International Congress of Biochemistry in Vienna (Austria).

E. V. Budnitskaia

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ABBREVIATIONS MOST FREQUENTLY ENCOUNTERED IN RUSSIAN BIO-SCIENCES LITERATURE

Abbreviation (Transliterated)	Significance
AMN SSSR	Academy of Medical Sciences, USSR
AN SSSR	Academy of Sciences, USSR
BIN	Biological Institute, Botanical Institute
FTI	Institute of Physiotherapy
GONTI	State United Sci-Tech Press
GOST	All Union State Standard
GRRRI	State Roentgenology, Radiology, and Cancer Institute
GTTI	State Technical and Theoretical Literature Press
GU	State University
I Kh N	Scientific Research Institute of Surgical Neuropathology
IL (IIL)	Foreign Literature Press
IONKh	Inst. Gen. and Inorganic Chemistry (N. S. Kurnakov)
IP	Soil Science Inst. (Acad. Sci. USSR)
ISN (Izd. Sov. Nauk)	Soviet Science Press
Izd.	Press
LEM	Laboratory for experimental morphogenesis
LENDVI	Leningrad Inst. of Dermatology and Venereology
LEO	Laboratory of Experimental Zoology
LIKhT	Leningrad Surgical Institute for Tuberculosis and Bone and Joint Diseases
LIPZ	Leningrad Inst. for Study of Occupational Diseases
LIPK	Leningrad Blood Transfusion Institute
Medgiz	State Medical Literature Press
MOPISh	Moscow Society of Apiculture and Sericulture
MVI	Moscow Veterinary Institute
MZdrav	Ministry of Health
MZI	Moscow Zootechnical Institute
LOKhO	Leningrad Society of Orthopedic Surgeons
NIIZ	Scientific Research Institute of Zoology
NINKhI	Scientific Research Institute of Neurosurgery
NIU	Scientific Institute for Fertilizers
NIUIF	Scientific Research Institute of Fertilizers and Insecticides
NIVI	Veterinary Scientific Research Institute
ONTI	United Sci. Tech. Press
OTI	Division of Technical Information
RBO	Russian Botanical Society
ROP	Russian Society of Pathologists
SANIIRI	Central Asia Scientific Research Institute of Irrigation
SANIISh	Central Asia Scientific Research Institute of Sericulture
TsNII	All-Union Central Scientific Research Institute
TsNTL	Central Scientific and Technical Laboratory
VASKhNIL	All-Union Academy of Agricultural Sciences
VIG	All-Union Institute of Helminthology
VIEM	All-Union Institute of Experimental Medicine
VIR	All-Union Institute of Plant Cultivation
VIUAA	All-Union Institute of Fertilizers, Soil Science, and Agricultural Engineering
VIZR	All-Union Institute of Medical and Pharmaceutical Herbs
VNIRO	All-Union Scientific Institute of Fishing and Oceanography
ZIN	Zoological Inst. (Acad. Sci. USSR)

Note: Abbreviations not on this list and not explained in the translation have been transliterated, no further information about their significance being available to us. - Publisher.

RUSSIAN JOURNALS FREQUENTLY CITED

[Biological Sciences]

Abbreviation*	Journal*	Translation
Agrobiol.	Agrobiologiia	Agrobiology
Akusherstvo i Ginekol.	Akusherstvo i Ginekologiia	Obstetrics and Gynecology
Antibiotiki	Antibiotiki	Antibiotics
Aptechnoe Delo	Aptechnoe Delo	Pharmaceutical Transactions
Arkh. Anat. Gistol. i Embriol.	Arkhiv Anatomii Gistologii i Embriologii	Archives of Anatomy, Histology, and Embryology
Arkh. Biol. Nauk SSSR	Arkhiv Biologicheskikh Nauk SSSR	Archives of Biological Science USSR
Arkh. Patol.	Arkhiv Patologii	Archives of Pathology
Biofizika	Biofizika	Biophysics
Biokhimiia	Biokhimiia	Biochemistry
Biokhim. Plodov i Ovoshchei	Biokhimiia Plodov i Ovoshchei	Biochemistry of Fruits and Vegetables
Bot. Zhur.	Botanicheskii Zhurnal	Journal of Botany
Biull. Eksptl. Biol. i Med.	Biulleten Eksperimentalnoi Biologii i Meditsiny	Bulletin of Experimental Biology and Medicine
Biull. Moskov. Obshchestva Ispytatelei Prirody, Otdel Biol.	Biulleten Moskovskogo Obshchestva Ispytatelei Prirody, Otdel Biologicheskii	Bulletin of the Moscow Naturalists Society, Division of Biology
Doklady Akad. Nauk SSSR	Doklady Akademii Nauk SSSR	Proceedings of the Academy of Sciences USSR
Eksptl. Khirurg.	Eksperimentalnaia Khirurgiia	Experimental Surgery
Farmakol. i Toksikol.	Farmakologiia i Toksikologiia	Pharmacology and Toxicology
Farmatsiia	Farmatsiia	Pharmacy
Fiziol. Rastenii	Fiziologiia Rastenii	Plant Physiology
Fiziol. Zhur. SSSR	Fiziologicheskii Zhurnal SSSR im. I. M. Sechenova	I. M. Sechenova Physiology Journal USSR
Gigiena i Sanit.	Gigiena i Sanitariia	Hygiene and Sanitation
Izvest. Akad. Nauk SSSR, Ser. Biol.	Izvestiia Akademii Nauk SSSR, Seriia Biologicheskaia	Bulletin of the Academy of Sciences USSR, Biology Series
Izvest. Tikhookeanskogo N. I. Inst. Rybnogo Khoz. i Okeanog.	Investiia Tikhookeanskogo N. I. Instituta Rybnogo Khoziaistva i Okeanografii	Bulletin of the Pacific Ocean Scientific Institute of Fisheries and Oceanography
Khirurgiia	Khirurgiia	Surgery
Klin. Med.	Klinicheskaia Meditsina	Clinical Medicine
Lab. Delo	Laboratornoe Delo(po Voprosam Meditsiny)	Laboratory Work (on Medical Problems)
Med. Parazitol.	Meditsinskaia Parazitologiia i Parazitarnye Bolezni	Medical Parasitology and Parasitic Diseases
Med. Radiol.	Meditsinskaia Radiologiia	Medical Radiology
Med. Zhur. Ukrain.	Medichnii Zhurnal Ukrainskii	Ukrainian Medical Journal
Mikrobiologiia	Mikrobiologiia	Microbiology
Mikrobiol. Zhur.	Mikrobiologichnii Zhurnal	Microbiology Journal
Nevropatol., Psikhiat. i Psikhogig.	Nevropatologiia, Psikhiatriia i Psikhogigiena	Neuropathology, Psychiatry and Psychohygiene
Ortoped., Travmatol. i Protez.	Ortopediia, Travmatologiia i Protezirovanie	Orthopedics, Traumatology and Prosthetics
Parazitol. Sbornik	Parazitologicheskii Sbornik	Parasitology Collection
Pediatriia	Pediatriia	Pediatrics
Pochvovedenie	Pochvovedenie	Soil Science
Priroda	Priroda	Nature
Problemy Endokrinol. i Gormonoterap.	Problemy Endokrinologii i Gormonoterapii	Problems of Endocrinology and Hormone Therapy
Problemy Gematol.	Problemy Gematologii i Perelivaniia Krovi	Problems of Hematology and Blood Transfusion
Problemy Tuberk.	Problemy Tuberkuleza	Problems of Tuberculosis
Sovet. Med.	Sovetskaia Meditsina	Soviet Medicine
Sovet. Vrachebny Zhur.	Sovetskii Vrachebnyi Zhurnal	Soviet Physicians Journals
Stomatologiia	Stomatologiia	Stomatology
Terap. Arkh.	Terapevticheskii Arkhiv	Therapeutic Archives
Trudy Gelmint. Lab.	Trudy Gelmintologicheskoi Laboratoriia	Transactions of the Helminthology Laboratory
Trudy Inst. Genet.	Trudy Instituta Genetiki	Transactions of the Institute of Genetics

* LIBRARY OF CONGRESS TRANSLITERATION SYSTEM.

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Abbreviation	Journal	Translation
Trudy Inst. Gidrobiol.	Trudy Instituta Gidrobiologiia	Transactions of the Institute of Hydrobiology
Trudy Inst. Mikrobiol.	Trudy Instituta Mikrobiologiia	Transactions of the Institute of Microbiology
Trudy Inst. Okean.	Trudy Instituta Okeanologiia, Akademii Nauk SSSR	Transactions of the Institute of Oceanology, Academy of Sciences, USSR
Trudy Leningrad Obshchestva Estestvoisp.	Trudy Leningrad Obshchestva Estestvoispytatelei	Transactions of the Leningrad Society of Naturalists
Trudy Vsesoiuz. Gidrobiol. Obshchestva	Trudy Vsesoiuznogo Gidrobiologicheskogo Obshchestva	Transactions of the All-Union Hydrobiological Society
Trudy Vsesoiuz. Inst. Eksptl. Med.	Trudy Vsesoiuznogo Instituta Eksperimentalnoi Meditsiny	Transactions of the All-Union Institute of Experimental Medicine
Ukrain. Biokhim. Zhur.	Ukrainskii Biokhimichnii Zhurnal	Ukrainian Biochemical Journal
Urologiia	Urologiia	Urology
Uspekhi Biokhimiia	Uspekhi Biokhimiia	Progress in Biochemistry
Uspekhi Sovremennoi Biol.	Uspekhi Sovremennoi Biologiia	Progress in Contemporary Biology
Vestnik Akad. Med. Nauk SSSR	Vestnik Akademii Meditsinskikh Nauk SSSR	Bulletin of the Academy of Medical Science USSR
Vestnik Khirurg. im. Grekova	Vestnik Khirurgii imeni Grekova	Grekov Bulletin of Surgery
Vestnik Leningrad. Univ. Ser. Biol.	Vestnik Leningradskogo Universiteta, Seriia Biologii	Journal of the Leningrad Univ., Biology Series
Vestnik Moskov. Univ., Ser. Biol. i Pochvov.	Vestnik Moskovskogo Universiteta, Seriia Biologii i Pochvovedeniia	Bulletin of the Moscow University, Biology and Soil Science Series
Vestnik Oftalmol.	Vestnik Oftalmologii	Bulletin of Ophthalmology
Vestnik Oto-rino-laringol.	Vestnik Oto-rino-laringologiia	Bulletin of Otorhinolaryngology
Vestnik Rentgenol. i Radiol.	Vestnik Rentgenologii i Radiologii	Bulletin of Roentgenology and Radiology
Vestnik Venerol. i Dermatol.	Vestnik Venerologii i Dermatologii	Bulletin of Venereology and Dermatology
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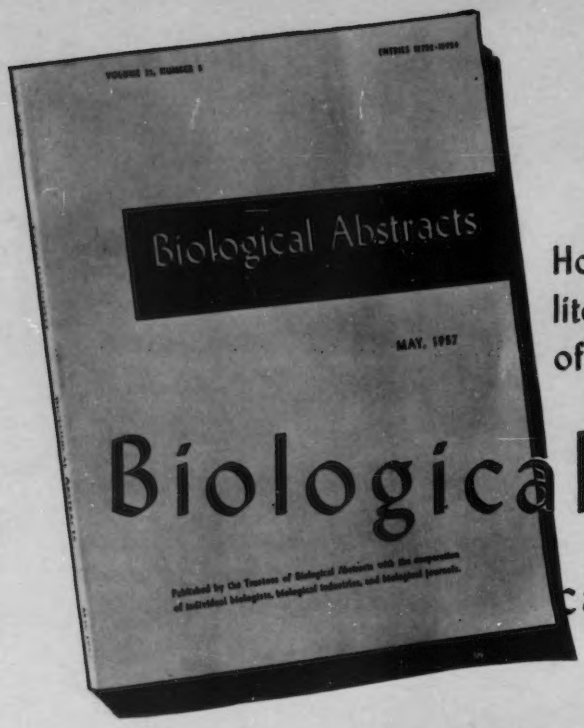

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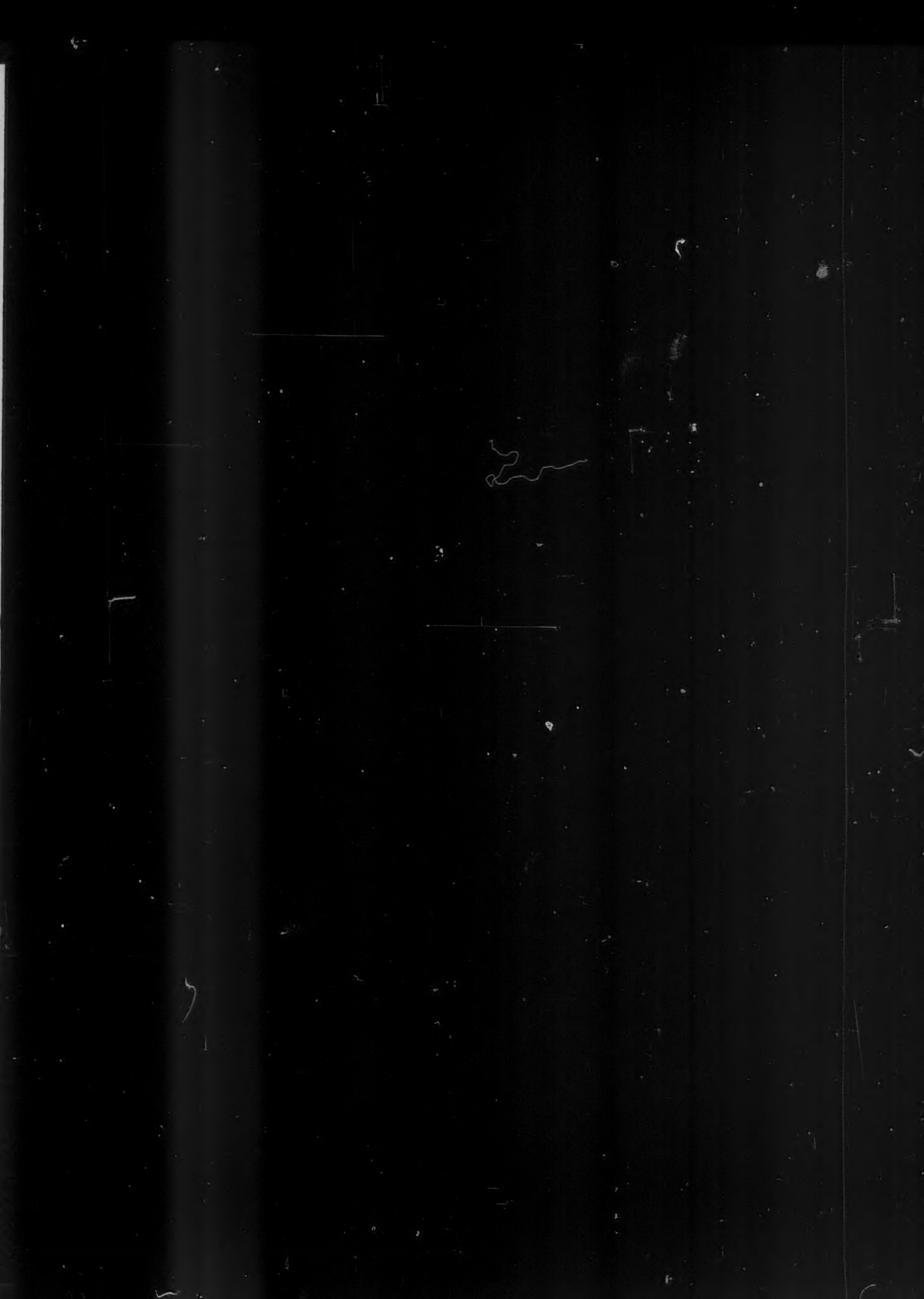
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